OCTOBER 1957, VOLUME 67, No. 2

UNIVERSITY OF MICHIGAN
OCT 29 1957
SCIENCE LIBRARY

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

A. G. OGSTON (*Chairman*) — H. J. ROGERS (*Deputy Chairman*)
E. P. ABRAHAM — L. W. MAPSON
H. R. V. ARNSTEIN — R. MARKHAM
S. R. ELSDEN — N. H. MARTIN
Q. H. GIBSON — R. J. PENNINGTON
C. H. GRAY — J. J. SCOTT
D. C. HARRISON — I. D. E. STOREY
C. LONG — W. V. THORPE
W. J. WHELAN

Secretary to the Board: F. CLARK

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W.1
AMERICAN BRANCH: 32 EAST 57TH STREET, NEW YORK 22, N.Y.

Price 25s. net (U.S.A. $4.25)

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in three volumes per year. Each volume consists of approximately 700 pages, published in four parts at intervals of one month.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are elegible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Honorary Secretaries, Professor F. L. Warren, Department of Biochemistry, London Hospital Medical College, Turner Street, London, E. 1, and Dr C. E. Dalgliesh, Postgraduate Medical School of London, Ducane Road, Hammersmith, London, W. 12, or from the Honorary Treasurer, F. A. Robinson, Esq., c/o Allen and Hanburys, Ltd., Bethnal Green, London, E. 2.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £4 per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, or to any bookseller. In the United States of America the *Journal* may be purchased from the Cambridge University Press American Branch, 32 East 57th Street, New York, 22 (subscription price $13.50 per volume).

Communications respecting the sale of single issues or back numbers of the *Journal*, Volumes 20–66, should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. Volumes 1–15 have been reproduced and are available from William Dawson & Sons, Ltd., 4 Duke Street, Manchester Square, London, W. 1, at the prices given below.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

Prices of back numbers of the *Journal*.

Volumes 1–15 complete.	£82. 10*s*. bound, £75 unbound
Separate volumes.	£6 each bound, £5. 10*s*. each unbound

Reduced terms for Volumes 1–15 are available to members of the Biochemical Society, who should apply to the Honorary Secretaries.

	In paper cover
Volumes 20–28.	£5 per volume of 6 parts at £1. 12*s*. each if sold separately
Volume 29.	Nos. 1, 2, 4, 6, 8–11. 16*s*. per number
Volume 33 Nos. 8–12 only.	16*s*. per number
Volume 33 Index.	2*s*. 6*d*. net
Volume 34 Nos. 4–7 & 12.	16*s*. per number
Volume 34 Nos. 8 & 9, 10 & 11.	£1. 12*s*. per double number
Volume 35.	£8 per volume of 12 parts
Volume 36.	£5 per volume of 12 parts at 10*s*. each if sold separately
Volume 37.	£5 per volume of 6 parts
Volume 39 Nos. 2 & 3.	24*s*. per number
Volume 40.	£5 per volume of 6 parts at £1 each if sold separately
Volumes 41–43.	£5 per volume of 4 parts at £1. 10*s*. each if sold separately
Volumes 44–47, 50–51.	£5 per volume of 5 parts at £1. 4*s*. each if sold separately
Volumes 52–54.	£4 per volume of 4 parts at £1 each if sold separately
Volume 55.	£5 per volume of 5 parts at £1 each if sold separately
Volumes 56–64.	£4 per volume of 4 parts at £1 each if sold separately
Volumes 65–66.	£5 per volume of 4 parts at £1. 5*s*. each if sold separately
Index to Volumes 11–20.	10*s*. net
Index to Volumes 21–30.	15*s*. net
Index to Volumes 31–40.	£1. 15*s*. net
History of the Biochemical Society.	2*s*. 6*d*. net

CONTENTS OF VOLUME 67, No. 2, 1957

(*All rights reserved*)

PAGE

A semi-microrespirometer. By J. W. RIDGE. (With two figures) 177

The biosynthesis of penicillin. 6. A study of the mechanism of the formation of the thiazolidine–β-lactam rings, using tritium-labelled cystine. By H. R. V. ARNSTEIN and J. C. CRAWHALL. (With three figures) 180

Some spontaneous reactions of *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate and related compounds in water and on storage, and their effects on the toxicological properties of the compounds. By D. F. HEATH and M. VANDEKAR. (With one figure) 187

The reactivation of cholinesterase after inhibition *in vivo* by some dimethyl phosphate esters. By M. VANDEKAR and D. F. HEATH. (With three figures) 202

Aspects of vitamin A deficiency in the rat. By F. W. HEATON, J. S. LOWE and R. A. MORTON. (With three figures) 208

Vitamin A deficiency in the domestic fowl. By J. S. LOWE, R. A. MORTON, N. F. CUNNINGHAM and J. VERNON. (With one figure) 215

Vitamin A deficiency in the rat, with and without added dietary cholesterol. By B. GREEN, J. S. LOWE and R. A. MORTON 223

Unsaponifiable constituents of kidney in various species. By J. S. LOWE, R. A. MORTON and J. VERNON 228

Cholesterol and liver vitamin A in male and female rats. By B. GREEN, A. A. HORNER, J. S. LOWE and R. A. MORTON 235

Enzymic oxidation of catechin to a polymer structurally related to some phlobatannins. By D. E. HATHWAY and J. W. T. SEAKINS. (With eight figures) 239

The characteristics of hexokinase from *Locusta migratoria* muscle. By M. KERLY and D. H. LEABACK. (With two figures) 245

The hexose specificity of hexokinase in brain from various species. By M. KERLY and D. H. LEABACK . 250

The identification of aldoheptose sugars. By D. A. L. DAVIES 253

Heterogeneity of normal adult human haemoglobin. By A. C. ALLISON and M. P. TOMBS. (With two figures) 256

Determination of creatine in biological fluids. By D. R. ANDERSON, C. M. WILLIAMS, G. M. KRISE and R. M. DOWBEN 258

Hormones and liver cytoplasm. 4. Ribonucleotides, ribonucleic acid synthesis and protein synthesis after adrenalectomy. By E. REID and B. M. STEVENS. (With six figures) 262

The biochemistry of aromatic amines. 4. *O*-Glucosiduronic acid derivatives of 2-naphthylamine. By E. BOYLAND and D. MANSON 275

The synthesis of fluorocitric acid and its inhibition in acetate. By R. A. PETERS and R. W. WAKELIN . 280

The formation of glycine and serine. The influence of the administration of glycine, DL-serine and other compounds on levels of tissue glycine and serine. By J. L. SIMKIN and K. WHITE 287

Determination of manganese in tissue extracts by means of formaldoxime. By W. BARTLEY, B. M. NOTTON and W. C. WERKHEISER. (With one figure) 291

Application of the carbazole reaction to the estimation of glucuronic acid and glucose in some acidic polysaccharides and in urine. By J. M. BOWNESS. (With two figures) 295

Latent phenolase in extracts of broad-bean (*Vicia faba* L.) leaves. Activation by acid and alkali. By R. H. KENTEN. (With two figures) 300

Sterol metabolism. 3. The distribution and transport of sterols across the intestinal mucosa of the guinea pig. By J. GLOVER and C. GREEN. (With three figures) 308

PAGE

The mechanism of the irreversible inhibition of rat liver monoamine oxidase by iproniazid (Marsilid). By A. N. Davison. (With four figures) 316

Comparative study of 'bile-salts'. 10. Bile salts of the King penguin, *Aptenodites patagonica*. By I. G. Anderson, G. A. D. Haslewood and I. D. P. Wootton 323

The effect of sheep-rumen contents on unsaturated fatty acids. By F. B. Shorland, R. O. Weenink, A. T. Johns and I. R. C. McDonald. (With one figure) 328

The ninhydrin reaction and its analytical applications. By H. Meyer. (With six figures) 333

Blood lipids. 1. Plasma lipids of the lactating cow: chromatography on silicic acid. By G. A. Garton and W. R. H. Duncan. (With one figure) 340

Blood lipids. 2. Plasma lipids of the lactating cow: fatty acid composition of the sterol esters and triglycerides. By A. K. Lough and G. A. Garton. (With five figures) 345

INDEX OF AUTHORS

PAGE

Allison, A. C. 256
Anderson, D. R. 258
Anderson, I. G. 323
Arnstein, H. R. V. 180

Bartley, W. 291
Bowness, J. M. 295
Boyland, E. 275

Crawhall, J. C. 180
Cunningham, N. F. 215

Davison, A. N. 316
Davies, D. A. L. 253
Dowben, R. M. 258
Duncan, W. R. H. 340

Garton, G. A. 340, 345
Glover, J. 308
Green, B. 223, 235
Green, C. 308

Haslewood, G. A. D. 323
Hathway, D. E. 239
Heath, D. F. 187, 202
Heaton, F. W. 208
Horner, A. A. 235

Johns, A. T. 328

Kenten, R. H. 300
Kerly, M. 245, 250
Krise, G. M. 258

PAGE

Leaback, D. H. 245, 250
Lough, A. K. 345
Lowe, J. S. 208, 215, 223, 228, 235

McDonald, I. R. C. 328
Manson, D. 275
Meyer, H. 333
Morton, R. A. 208, 215, 223, 228, 235

Notton, B. M. 291

Peters, R. A. 280

Reid, E. 262
Ridge, J. W. 177

Seakins, J. W. T. 239
Shorland, F. B. 328
Simkin, J. L. 287
Stevens, B. M. 262

Tombs, M. P. 256

Vandekar, M. 187, 202
Vernon, J. 215, 228

Wakelin, R. W. 280
Weenink, R. O. 328
Werkheiser, W. C. 291
White, K. 287
Williams, C. M. 258
Wootton, I. D. P. 323

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

Inhibition of the alanine–α-oxoglutaric acid transaminase of pig-heart muscle by cyclic hydrazides. By R. Marian Hicks and J. Cymerman-Craig

The blocking activity of sympathomimetic amines on adrenaline hyperglycaemia. By Adrienne Gourju and A. Lawson

The synthesis of lipids in the livers of rats treated with pituitary growth hormone. By A. L. Greenbaum and R. F. Glascock

Analysis of tissue constituents. Commercial ox-muscle extract. By T. Wood and A. E. Bender

Studies on the aerobic degradation of glucose by *Saccharomyces cerevisiae*. By N. R. Eaton and H. P. Klein

The accumulation of iodide by *Fucus ceranoides*. By H. G. Klemperer

Studies in the biochemistry of micro-organisms. 103. Metabolites of *Alternaria tenuis* Auct.: culture filtrate products. By T. Rosett, R. H. Sankhala, C. E. Stickings, M. E. U. Taylor and R. Thomas

The conversion of casein into microbial proteins in the rumen. By I. W. McDonald and R. J. Hall

Essential role of thiol groups in aldehyde dehydrogenases. By A. O. M. Stoppani and C. Milstein

Reaction of ninhydrin in acid solution with straight-chain amino acids containing two amino groups and its application to the estimation of αε-diaminopimelic acid. By Elizabeth Work

Liver and brain mitochondria. By W. N. Aldridge

An electronic colloid osmometer and an assessment of its accuracy. The molecular weight of bovine plasma albumin. By D. S. Rowe and M. E. Abrams

The molecular weights of the proteins of normal and nephrotic sera and nephrotic urine, and a comparison of selective ultrafiltrates of serum proteins with urine proteins. By D. S. Rowe

The transformation of gallates into ellagate. By D. E. Hathway

The biosynthesis of sucrose. By J. F. Turner

Formation of 3-hydroxybutan-2-one and butane-2:3-diol by pig-heart preparations and its relationship to oxidation of pyruvate. By J. Dawson, R. P. Hullin and Marjorie Walker

β-Glucuronidase activity during the early development of *Xenopus laevis* embryos. By F. Billett

The phosphoesterase of pea plants (*Pisum sativum* L.). By W. S. Pierpoint

Calcium and magnesium metabolism in calves. Plasma levels and retention in milk-fed calves. By R. H. Smith

A new type of esterase in hog-kidney extract. By F. Bergmann, Ruth Segal and Sara Rimon

The oxidative activity of particulate fractions from mosquitoes. By O. Gonda, A. Traub and Y. Avi-Dor

Effects of 5-halogenated uracils on the growth of *Escherichia coli* and their incorporation into deoxyribonucleic acids. By D. B. Dunn and J. D. Smith

The assimilation of amino acids by bacteria. 24. Inhibitors of incorporation of glycine in disrupted staphylococcal cells. By E. F. Gale and Joan P. Folkes

The solubility and fractionation of β_1-lactoglobulin. By M. P. Tombs

The isolation of inositol monophosphate from liver. By G. Hübscher and J. N. Hawthorne

THE BIOCHEMICAL JOURNAL

NOTES FOR CONTRIBUTORS

Papers submitted for publication in the *Biochemical Journal* should be written concisely. The sections below concerning the preparation of the typescript give only general indications. Authors are urged to consult the more detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions,* which was published in May 1957 (*Biochem. J.* 1957, 66, 1); also *Notes on Preparation of Illustrations,* which was published in January 1956 (*Biochem. J.* 1956, 62). Copies of these pamphlets may be obtained from the Editorial Office (*Biochemical Journal*), The Lister Institute, Chelsea Bridge Road, London, S.W. 1, price 1*s.* 6*d.* (*Suggestions to Authors*) and 1*s.* (*Notes on Preparation of Illustrations*) post free.

Strict observance of the requirements of the *Biochemical Journal* will shorten the period between the receipt of a paper and its publication.

Communications. Papers submitted for publication should be sent to the Secretary to the Editorial Board (*Biochemical Journal*), The Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1.

Abstracts. Authors should submit with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences*. This abstract will not appear in the *Biochemical Journal* but will be edited before being passed for publication in the *Abstracts*.

The abstract should outline as briefly as possible the results and definitive conclusions of the work submitted. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet in the following form: title; name(s) of author(s); *Biochem. J.* (space for year, volume and page reference); address (for reprint applications); abstract. For example:

> The metabolism of short-chain fatty acids in the sheep. 4. The pathway of propionate metabolism in rumen epithelial tissue. R. J. Pennington and T. M. Sutherland. *Biochem. J.* 1956, **63**, 618–628 (Rowett Research Institute, Bucksburn, Aberdeenshire)—(Abstract follows).

Reprints. Where at least one author of a paper is a member of the Biochemical Society, twenty-five reprints are supplied free of cost. An author may purchase additional reprints if he notifies the Press on the appropriate form immediately the proof of the paper is received. Communications about the purchase of reprints should be addressed to the University Press, Cambridge.

General. It is the policy of the *Biochemical Journal* to publish papers in all fields of biochemistry—plant, animal and microbiological—provided that they describe results which make a new and fundamental contribution to biochemical knowledge, or that they describe methods applicable to biochemical problems. Submission of a paper to the Editorial Board will be held to imply that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Biochemical Journal* it will not be published elsewhere in the same form, either in English or in any other language, without the consent of the Editorial Board.

Contributors who reside abroad may nominate somebody in Great Britain who is willing to correct the proofs of their papers. Proofs are also sent to all authors residing abroad, if necessary by airmail, whether or not they have nominated a proof reader

p.t.o.

in Great Britain; if these are returned immediately, it will normally be possible to incorporate corrections in the final proof. The method of correcting proofs given in B.S. 1219 or B.S. 1219C (obtainable from the British Standards Institution) is preferred.

Papers should be headed by an informative title, by the names of the authors and by the name and address of the Laboratory where the work was performed. Female authors should use one given name and the surname, and male authors should use initials and surname only. Descriptive material about the author, e.g. Beit Memorial Fellow, or details of financial support, should appear as a footnote on the first page or, preferably, in the acknowledgements at the end of the paper.

Typescripts should bear the name and address of the person to whom the proof of the paper is to be sent, and should give also a shortened version of the title, not exceeding forty-five letters and spaces in length, suitable for a running title in the published pages of the work.

If a paper that has been returned to an author for revision is not resubmitted within six months, it will be deemed to be a new paper and the date of receipt altered accordingly. A revised paper containing a significant amount of new material will also be redated.

A paper should be written only when a piece of work is rounded off. Preliminary or abortive experiments should not be described.

It would help the editors if the author, when submitting a paper which is part of a series, would enclose reprints of the immediately preceding parts.

Form of papers submitted for publication. Papers should be in double-spaced typing on sheets of uniform size with wide margins. Top copies only should be submitted. The paper should be written in English.

The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author, who should first consult the detailed *Suggestions to Authors, Chemical Nomenclature and Abbreviations, Symbols, Usages and Conventions.* Authors should also refer to a current issue of the *Journal* in order to make themselves familiar with the typographical conventions, use of cross-headings, lay-out of tables, citation of references, etc. The need for editorial revision of a badly prepared typescript will lead to delay in publication. Papers on specialized subjects should be presented so that they are intelligible to the ordinary reader of the *Journal*. Sufficient information should be included to permit repetition of the experimental work.

Generally, papers should be divided clearly into sections, as follows: (*a*) introduction, containing the reasons for doing the work; (*b*) Experimental methods: with chemical papers the experimental part may appear towards the end, but otherwise should follow the introduction; (*c*) Results: these should be given concisely; the use of both tables and figures to illustrate the same results will only rarely be permitted; only illustrative protocols should be included; (*d*) Discussion: it is desirable that the presentation of the results should be separated from the discussion of their significance; this section should be strictly limited to discussion, and should not recapitulate results; (*e*) a Summary, about 3 % of the length of the paper: the paragraphs of the Summary should be numbered; (*f*) acknowledgements; (*g*) References. The arrangement suggested for sections (*b*)–(*d*) is not binding on authors; other ways of arrangement are sometimes more suitable.

The *Biochemical Journal* uses as a standard for spelling the *Concise Oxford Dictionary of Current English* (Oxford: Clarendon Press).

Illustrations. Diagrams that do not conform with the directions given in *Notes on Preparation of Illustrations* may have to be redrawn by the Press and the expense charged to the author. Legends and captions should be written so that the general meaning of each illustration can be understood without reference to the text, and so that the exact experimental conditions used to obtain the results illustrated are made clear. Illustrations requiring reproduction as half-tone plates should be avoided whenever possible. Photographs or drawings of paper chromatograms, particularly one-dimensional, are not generally published.

Tables. Tables should have headings which make their general meaning comprehensible without reference to the text. Conditions specific to the particular experiment should be stated. Reference to the text for general experimental methods is permissible provided that there is no ambiguity. The units in which the results are expressed, e.g. g./100 ml., should be given at the top of each column, and not repeated on each line of the table.

Tables should be typed on separate sheets and their approximate position in the text indicated. Words or numerals should be repeated on successive lines: 'ditto' or '„' are not to be used.

Footnotes. These should be avoided in the text as far as possible.

A Semi-Microrespirometer

By J. W. RIDGE
Ophthalmological Research Unit (Medical Research Council), Institute of Ophthalmology, Judd Street, London, W.C. 1

(*Received* 3 *October* 1956)

A sensitivity greater than that of the standard Barcroft apparatus may be obtained by scaling down the apparatus. Various modifications to such an apparatus, designed to reduce the errors inherent in the estimation of small pressure changes, have been reported. Perhaps the most widely adopted of these is the introduction, on the reaction chamber side, of a micrometer syringe by means of which the pressure in the reaction vessel can be equilibrated with that in the compensation vessel (e.g. Adam, 1920; Tyler & Berg, 1941; Scholander, 1942; Dixon, 1951). In general, the reaction and compensation vessels were of the same capacity.

The purpose of the present paper is to describe a micro-Barcroft apparatus in which the compensation vessel is several times larger than the reaction chamber and the micrometer syringe is placed on the compensation side. In this way the pressure changes in the reaction vessel are magnified approximately in the ratio of the capacities of the two chambers, and can be estimated with a smaller error.

APPARATUS

In Fig. 1, *A* is the reaction chamber, which had a capacity of about 1 ml. It had two compartments, the smaller for the tissue, the larger for the KOH, and was connected to the manometer *B* (Pyrex capillary, bore 0·4 mm.) by small-bore, thick-walled polythene tubing. The reaction chamber could thus be shaken independently of the rest of the apparatus. The manometric fluid was the fraction of commercial paraffin boiling between 160° and 190°, coloured with a little Sudan III. This fluid has a low vapour pressure and a high mobility. *D* is a bridge and three-way tap to equalize the pressure on either side of the manometer after temperature equilibration had been attained. The compensation chamber *E* was connected to the manometer by a B14 standard ground-glass joint allowing compensators of different sizes to be fitted. The apparatus was connected through a levelling bulb *F* and a length of small-bore Portex (vinyl–vinylidene chloride co-polymer) tubing (Portland Plastics Ltd., Hythe, Kent) to an Agla syringe and micrometer *G*. The syringe and Portex tube were filled with mercury, initially, up to one of two datum lines; the upper (just above *F*) was used when gas uptake was expected, the lower (just below *F*) when gas was

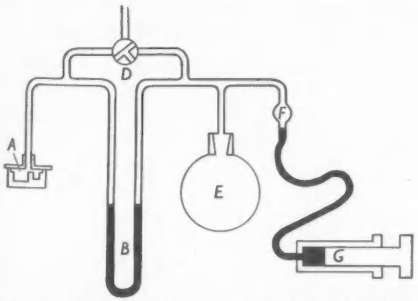


Fig. 1. Diagram of the apparatus. For explanation see text.

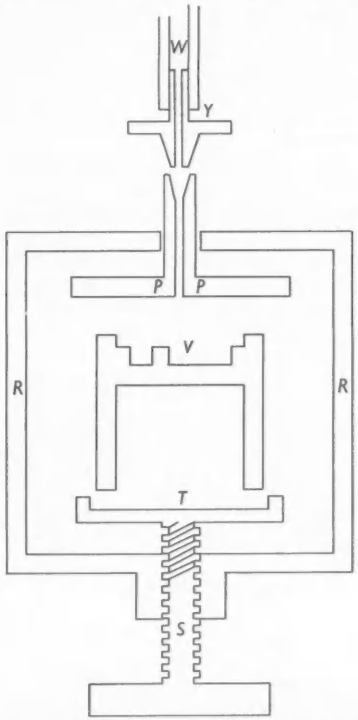


Fig. 2. Section through the reaction chamber assembly. For explanation see text.

evolved. The whole apparatus, with the exception of the syringe and the micrometer, was immersed in a water bath. At 30°, temperature equilibration was attained in 20 min.

The details of the reaction chamber assembly are given in Fig. 2. The chamber V was drilled from Perspex rod (1 in. diam.) and excess of material was removed to hasten temperature equilibration. The chamber was carried on the brass table T which could be raised or lowered by means of the screw S. A raised rim on T ensured accurate and reproducible alignment of the chamber with the upper brass plate P. The whole was held in the rigid brass frame R. The polythene tube W was joined to the reaction-chamber assembly by the connector Y. Thus the whole assembly could be rapidly dismantled and detached from the apparatus for cleaning and charging. These operations had no noticeable effect on the calibration of the instrument.

The menisci of the manometric fluid were viewed through a simple reading microscope (5 in. objective and $\times 10$ cross-line micrometer eyepiece) and kept on the hairline by manipulation of the micrometer. A reading could be taken in a few seconds; duplicate readings agreed well, the agreement being dependent on the capacity of the compensation vessel. As a rule they did not differ by more than ± 3 graduations with a compensation vessel of 100 ml., and by ± 1 graduation with one of 10 ml. capacity.

The experiments for which this apparatus was designed did not require the instrument to be filled with a gas mixture differing from that of air. For that reason no provision was made for filling the system with any other gas mixture. However, the apparatus is capable of modification without affecting the underlying principles.

THEORY

Let x = the amount of gas evolved, in μl. at s.t.p. (if the gas is absorbed x will be negative);

V = total volume of the reaction vessel;

V_F = the liquid volume in the reaction vessel;

V_G = the gas volume in the reaction vessel;

ΔP = the change of pressure in the reaction vessel (if gas is absorbed ΔP will be negative);

T = the absolute temperature of the water bath;

P = the atmospheric pressure;

p = the vapour pressure of water vapour at T;

P_0 = the standard pressure;

α = Bunsen's absorption coefficient of the gases in the reaction vessel (a composite figure depending on the nature and partial pressures of the gases concerned);

V_{Hg} = volume of mercury required to adjust manometer levels (if gas is absorbed V_{Hg} will be negative).

The same symbols with dashes are used for the compensation vessel.

Then the amount of gas initially present in the reaction chamber is

$$V_G \frac{273}{T} \frac{(P-p)}{P_0},$$

and the final amount after the manometer has been adjusted is

$$V_G \frac{273}{T} \frac{(P-p+\Delta P)}{P_0},$$

whence

$$x = V_G \frac{273}{T} \frac{(P-p+\Delta P)}{P_0} - V_G \frac{273}{T} \frac{(P-p)}{P_0}$$

$$= V_G \frac{273}{T} \frac{\Delta P}{P_0}. \quad (1)$$

The amount of gas initially present on the compensating side is

$$V'_G \frac{273}{T} \frac{(P-p)}{P_0}, \quad (2)$$

and that finally present

$$(V'_G - V_{Hg}) \frac{273}{T} \frac{(P-p+\Delta P)}{P_0}. \quad (3)$$

Since no gas is absorbed or evolved in the compensating vessel, (2) = (3), whence

$$\Delta P = \frac{V_{Hg}(P-p)}{V'_G - V_{Hg}}.$$

Substituting for ΔP in (1)

$$x = \frac{V_G}{(V'_G - V_{Hg})} \frac{273}{T} \frac{(P-p)}{P_0} V_{Hg}. \quad (4)$$

During the experiment the partial pressures of the gases in both vessels change, resulting in secondary changes in pressure due to gases either diffusing out of or dissolving in the liquids in the vessels. This effect is not taken account of in equation (4). The respective pressure changes are:

$\frac{V_F \alpha \Delta P}{P_0}$ in the reaction chamber and $\frac{V'_F \alpha' \Delta P}{P_0}$ in the compensation chamber.

Rather than introduce correction factors incorporating these quantities, it was considered preferable to adjust the ratio of the liquid volumes in both vessels in such a way that the secondary pressure changes in the two vessels cancel out. This can be done by arranging for V_F and V'_F to be such that the ratio of the pressure changes in the two vessels equals the ratio of the gas volumes; i.e.

$$\frac{V_F \alpha}{V'_F \alpha'} = \frac{V_G}{V'_G} = \frac{V_G}{V' - V'_F},$$

whence

$$V'_F = \frac{V_F \alpha V'}{V_F \alpha + V_G \alpha'}. \quad (5)$$

It may be noted that if α and α' are equal, or if they differ by a negligibly small amount, equation (5) reduces to

$$\frac{V'_F}{V_F} = \frac{V'}{V}.$$

Thus in these circumstances the identity to be satisfied is

$$\frac{V'_F}{V_F} = \frac{V'}{V} = \frac{V'_G}{V_G}.$$

This treatment is still not rigid since the adjustment of the liquid volumes is based on fixed gas volumes in both vessels, while the essence of the method is that V'_G can be varied at will. From equation (4), it is seen that for a given experiment

$$x = \frac{k V_G V_{Hg}}{V'_G - V_{Hg}},$$

where k has a value of about 0·8. Therefore

$$V_{Hg} = \frac{x V'_G}{k V_G + x}.$$

Expressing V_{Hg} as a percentage of V'_G,

$$\frac{100V_{Hg}}{V'_G} = \frac{100x}{kV_G + x}.$$

Thus the percentage error introduced by neglecting V_{Hg} compared with V'_G is independent of the ratio V_G/V'_G but can be conveniently related to V_G. Furthermore, V_G can be so fixed that the percentage error introduced above is kept down to a negligible value.

RESULTS

Two series of tests have been carried out on the apparatus. In the first, the observed volume of CO_2 liberated by the action of acid on Na_2CO_3 was compared with the theoretical volume. A volume (12·7 μl.) of Na_2CO_3 solution of known concentration was placed, as a single drop, in the large compartment of the reaction chamber along with a drop (also 12·7 μl.) of N-HCl and a lead shot coated with Perspex. The solutions were mixed by shaking the reaction chamber so that the lead shot mixed the reagents. The recoveries were quantitative (Table 1).

In the second test the rate of O_2 uptake by yeast suspensions as measured in the micro-apparatus was compared with the rate as measured in the standard Warburg apparatus. The results are recorded in Table 2.

Table 1. *Evolution of carbon dioxide from sodium carbonate*

Carbon dioxide was liberated by the action of N-HCl (12·7 μl.) on Na_2CO_3 solutions (12·7 μl.) of various concentrations.

V'_G/V_G ratio	Vol. of CO_2 at s.t.p. (μl.)		Observed as % of theoretical
	Theoretical	Observed	
100	0·28	0·30	107
	0·64	0·64	100
	1·26	1·26	100
	1·64	1·60	98
10	2·67	2·67	100
	5·04	4·92	98
	10·80	10·54	98
	15·49	15·62	101

Table 2. *Respiration of a yeast suspension*

Rates of O_2 uptake by two different samples of yeast were measured by the standard Warburg apparatus and on the microrespirometer ($V'_G/V_G = 10$).

Sample	Wt. of yeast (mg.)	Time (min.)	O_2 uptake (μl./mg. wet wt./hr.)	
			Micro	Warburg
A	0·52	20	−59	—
	0·49	20	−58	—
	3·8	30	—	−61
	6·5	15	—	−64
B	0·83	20	−44	—
	0·81	20	−39	—
	4·1	20	—	−42
	5·9	20	—	−43

DISCUSSION

The main purpose of this paper is to draw attention to the principle of the apparatus, but the following points might prove of use in the design and construction of a similar apparatus.

(1) Changes in the bath temperature which affect both vessels simultaneously and to the same extent will not upset their pressure relationship (except in so far as one side might take longer to reach equilibrium than the other), but differential temperature effects will drastically alter the pressure relationship. Such effects can be minimized, at the design stage, by mounting the reaction and compensation vessels as close together as possible and providing for great accuracy and sensitivity of temperature control.

(2) Although there seems to be no theoretical limit to the size of the ratio V'_G/V_G, it was found in practice that the apparatus became unstable when the ratio was greater than about 130:1 and the index fluid moved spontaneously. This movement was always in the same direction, namely consistent with an increase of pressure in the compensation chamber. It appears to require 4 or 5 days for the apparatus to settle down. This drift, the cause of which has not been definitely established, has been noticed by earlier authors (e.g. Adam, 1920; Schmitt, 1933; Tyler & Berg, 1941).

(3) A further limit to the magnitude of the ratio V'_G/V_G is imposed by the fact that as the ratio increases so does V_{Hg} for a given value of x. Thus in the present apparatus, at a ratio greater than about 150:1, the greater theoretical sensitivity of the apparatus was outweighed by a marked sluggishness of response to the addition or withdrawal of mercury.

The maximum practical value for V'_G/V_G in the present case was about 100 and the optimum about 50.

SUMMARY

1. A micro-Barcroft apparatus is described in which the reaction and compensation vessels are of different capacities and in which the pressures on either side of the manometer are equilibrated by altering the volume on the compensating side.

2. The theory of the apparatus is given.

REFERENCES

Adam, N. K. (1920). *Biochem. J.* **14**, 679.

Dixon, M. (1951). *Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processses*, 3rd ed., p. 6. Cambridge University Press.

Schmitt, F. O. (1933). *Amer. J. Physiol.* **104**, 303.

Scholander, P. F. (1942). *Rev. sci. Instrum.* **13**, 32.

Tyler, A. & Berg, W. E. (1941). *Science*, **94**, 397.

The Biosynthesis of Penicillin

6. A STUDY OF THE MECHANISM OF THE FORMATION OF THE THIAZOLIDINE–β-LACTAM RINGS, USING TRITIUM-LABELLED CYSTINE*

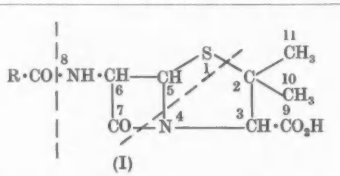
BY H. R. V. ARNSTEIN AND J. C. CRAWHALL
National Institute for Medical Research, Mill Hill, London, N.W. 7, and Department of Chemistry and Biochemistry, The Medical College of St Bartholomew's Hospital, London, E.C. 1

(*Received* 26 *February* 1957)

Previous work has established that part of the penicillin molecule (I), consisting of the side-chain nitrogen atom, $N_{(8)}$, the β-lactam carbon atoms, $C_{(5)-(7)}$, and the sulphur atom, is specifically derived from L-cysteine (Arnstein & Grant, 1954*b*), whereas the carbon chain of the penicillamine moiety is derived from valine (Stevens, Vohra & DeLong, 1954; Arnstein & Grant, 1954*a*). It is not yet certain, however, whether L- or D-valine is specifically used for penicillin biosynthesis (Arnstein & Clubb, 1957).

The mechanism by which cysteine and valine give rise to the fused thiazolidine–β-lactam ring structure is still obscure. Phenylacetyl-L-cysteine (II) (Arnstein, Clubb & Grant, 1954), β-hydroxyvaline (III) (Arnstein & Clubb, 1957) and ββ-dimethyllanthionine (IV) (Stevens, Vohra, Moore & DeLong, 1954) appear to be excluded as intermediates (for a recent review, see Arnstein & Grant, 1956) and the initial reaction, therefore, may be formation of cysteinylvaline and thus of the β-lactam–peptide bond.

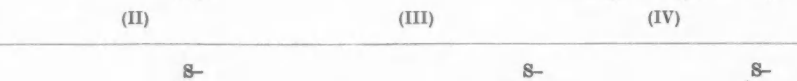
The present experiments deal specifically with the biosynthesis of the carbon–nitrogen bond common to the thiazolidine and β-lactam rings of penicillin; although this problem is, of course, connected to some extent with the nature of the initial reaction between cysteine and valine, which has not yet been fully elucidated, it has been possible to study certain of its aspects independently. In particular, two mechanisms involving possible dehydropeptide derivatives of cysteine and valine have been considered. Thus the β-lactam ring could be formed by addition of the hydrogen atom of the peptide bond to an αβ-dehydrocysteine derivative (Fig. 1, *A*) or by addition of one of the



(I)

R = $C_6H_5 \cdot CH_2 \cdot$ in benzylpenicillin. The broken lines indicate the metabolic origin of the thiazolidine–β-lactam ring structure from cysteine and valine.

* Part 5: Arnstein & Clubb (1957).



(II) (III) (IV)

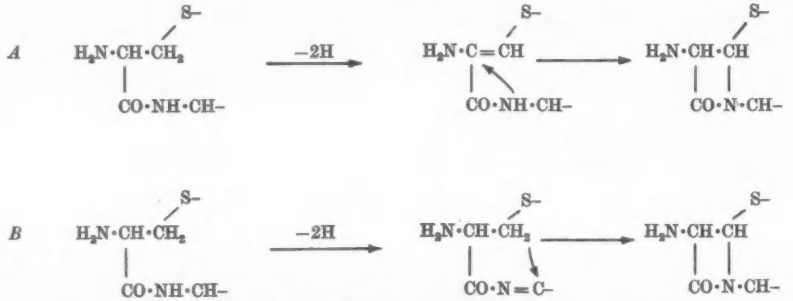


Fig. 1. Possible mechanisms of β-lactam formation.

β-hydrogen atoms of cysteine to an imino derivative of valine (Fig. 1, *B*). These alternatives have been investigated with cystine containing tritium in the α- or β-position. Reaction *A* would involve loss of isotopic hydrogen from the α-position of cystine, whereas mechanism *B* would lead to incorporation of labelled hydrogen from the β-position of cysteine into the α-position of the penicillamine moiety of penicillin. The results of the present work show, however, that the α-hydrogen atom of cystine is stable and no migration of the β-hydrogen atoms of cysteine during penicillin biosynthesis was detected.

A preliminary communication of this work was given at the 357th meeting of the Biochemical Society (Arnstein & Crawhall, 1957).

EXPERIMENTAL

Fermentations

Penicillium chrysogenum WIS 51–20 was used in all experiments and full details of the fermentation apparatus, culture medium and estimation of penicillin by bioassay have been given in a previous paper (Arnstein & Grant 1954*b*). Details of individual experiments are summarized in Table 1.

The experiment with washed mycelium (see Table 2) was carried out essentially as described by Halliday & Arnstein (1956).

Isolation and degradation methods

Penicillin. Penicillin was extracted from the acidified culture medium and concentrated by successive extractions between buffer and ether (Smith & Hockenhull, 1952). Finally the *N*-ethylpiperidine salt was isolated and recrystallized to constant radioactivity (see Arnstein & Grant, 1954*b*). Degradation of the *N*-ethylpiperidine salt was carried out as described by Arnstein & Clubb (1957), penicillamine being isolated as the *iso*propylidene derivative and penilloaldehyde as the 2:4-dinitrophenylhydrazone. The degradation of penilloaldehyde followed the procedure described earlier (Arnstein & Grant, 1954*a*), except that glycine was degraded to formaldehyde and CO_2 without prior isolation.

Amino acids from mycelium. In Expt. no. 1*a*, the mycelium was extracted with ethanol–ether (3:1, v/v), 5% (w/w) trichloroacetic acid, water, ethanol and ether, dried (dry wt., 5·9 g.) and hydrolysed with 6N-HCl (cf. Arnstein & Grant, 1954*b*). The hydrolysate was treated with Br_2 to oxidize cystine to cysteic acid, the acidic amino acids were separated by chromatography on Deacidite FF resin (Permutit Co. Ltd., London) in the acetate form and the non-acidic fraction from this column was further chromatographed on Zeo-Karb 225 resin (Permutit Co. Ltd., London), with essentially the same procedure as described by Hirs, Moore & Stein (1954) for the analogous Dowex 1 and Dowex 50 resins.

Cysteic acid was isolated as the Ba salt by evaporating the appropriate fractions from the Deacidite FF column to dryness, and adding dilute $Ba(OH)_2$ soln. till the pH was 6, followed by increasing quantities of ethanol. The following fractions were thus obtained, the first being precipitated by an equal volume of ethanol: (1) 19·6 mg., 107 counts/min.; (2) 10·1 mg., 265 counts/min.; (3) 10·6 mg., 291 counts/min. Fractions nos. 2 and 3 were combined and Ba^{2+} ions removed as $BaSO_4$ by adding dilute H_2SO_4. The residual aqueous solution was evaporated almost to dryness and the cysteic acid was crystallized by adding ethanol. The radioactivity of this material (384 counts/min.) was in good agreement with the average radioactivity of the Ba salt of fractions no. 2 and 3, when due allowance is made for the difference in molecular wt.

Glutamic acid and aspartic acid were isolated by evaporating the relevant fractions to dryness and crystallizing the residue from aqueous ethanol.

The remaining amino acids in Table 3 were isolated from the appropriate fractions of the Zeo-Karb 225 column, the hydrochlorides being converted into the free amino acids by means of a small column of Zeo-Karb 215 resin (Arnstein & Stanković, 1956). The amino acids were crystallized from aqueous ethanol.

Radioactivity estimations

Radioactive ^{14}C. Samples of 'infinite thickness' were mounted on 1 cm.^{2} polythene disks (Popják, 1950) and counted with a thin end-window Geiger–Müller tube.

Tritium. Compounds described in the section on the synthesis of labelled cystine were counted at St Bartholomew's Hospital Medical College on the windowless counter described by Banks, Blow & Francis (1956), which has an efficiency of about 0·3% when 10 mg. samples are counted after dilution with an equal wt. of graphite. All other samples were counted at the National Institute for Medical Research with the windowless flow-type Geiger counter described by Garrow & Piper (1955), which was found to have a similar efficiency. Samples were mixed with an equal wt. of graphite (Banks, Crawhall & Smyth, 1956) and mounted on a 1 cm.^{2} stainless-steel disk. The consistency of tritium assays on the latter counter was checked by comparing a sample of DL-[α-^{3}H]cystine with a poly[^{14}C]methyl methacrylate reference standard of specific radioactivity 1 μc/g., obtained from the Radiochemical Centre, Amersham, Bucks. Over a period of about 4 weeks, the average of ten determinations of the [α-^{3}H]cystine was found to be 426·2 counts/min., the range being 383·8–457·5. The maximum

Table 1. *Experimental details of fermentations with tritium-labelled cystine*

All fermentations contained 1·5 l. of medium.

Expt. no.	Precursor added	Amount of precursor added (mg.) and times of addition after inoculation (hr.) in parentheses	Yield of penicillin (i.u./ml.)
1*a*	DL-[α-^{3}H]Cystine	84 (1·5), 84 (26), 84 (50), 68 (75), 68 (102)	265 at 114 hr.
1*b*	DL-[α-^{3}H]Cystine	23 (44), 23 (61), 16·5 (68)	157 at 107 hr.
3	DL-[β-^{3}H]Cystine	26·5 (46), 26·5 (70), 26·5 (94)	174 at 110 hr.

deviation from the mean is thus approximately ±10%. Although this figure is considerably greater than that reported by Banks, Crawhall & Smyth (1956) for triplicate assays counted on the same day (±2%), it is acceptable in the present work, since it was necessary to differentiate only between retention and complete or almost complete loss of tritium in the reactions studied.

All counts have been corrected for dilution of sample by graphite and are calculated for a counting rate of the ^{14}C-standard equal to 1000 counts/min. The radioactivity of substances counted at St Bartholomew's Hospital Medical College has been calculated on the same basis, following a direct comparison of the above-mentioned sample of [α-^{3}H]cystine.

Syntheses of labelled cystine

The syntheses of [^{3}H]cystine from ^3H_2O (obtained from the Atomic Energy Research Establishment, Harwell, Berks.) are outlined in Fig. 2. For preparation of DL-[β-^{14}C]cystine see Arnstein & Crawhall (1953).

DL-[α-^{3}H]*Cystine*

(*a*) *Diethyl α-acetamido-β-benzylthiomethylmalonate* (*V*). A solution of Na (4·6 g.) in dry ethanol (100 ml.) was added to ethyl acetamidomalonate (43·4 g.) in dry ethanol (100 ml.). The mixture was evaporated to dryness *in vacuo*, dry toluene (50 ml.) was added and the evaporation was repeated. Dry toluene (100 ml.) and benzylthiomethyl chloride (34·6 g.) (Wood & du Vigneaud, 1939) were added and the mixture was boiled under reflux for 2·5 hr. After cooling, NaCl was removed by filtration and the filtrate evaporated to dryness, when a crystalline product was obtained. After it had been washed with light petroleum (b.p. 60–80°), the material (54 g., 76·5%) had m.p. 54–59°. Hydrolysis of a small portion of this material with 4 N-HCl at 105° for 18 hr. gave a mixture which was shown to contain approximately 50% of *S*-benzylcysteine (R_F 0·4) and glycine (R_F 0·1) by paper chromatography (pentan-1-ol–pyridine–water, 7:7:6, by vol.). Recrystallization of the crude product, first from methanol by addition of water, then from light petroleum (b.p. 60–80°), yielded 29 g. of *diethyl α-acetamido-β-benzylthiomethylmalonate*, m.p. 92° (Found: C, 57·9; H, 6·8; N, 3·75; S, 9·1. $C_{17}H_{23}O_5NS$ requires C, 57·8; H, 6·6; N, 4·0; S, 9·1%).

S-*Benzyl*-[α-^{3}H]*cysteine*. The diester (V) (1·05 g., 3 m-moles) was dissolved in 0·1 N-KOH in ethanol (60 ml.). After 24 hr. the ethanol was removed at 35° under reduced pressure and the residue was dried in a vacuum desiccator. Tritiated acetic acid, prepared from 2·4 ml. of acetic anhydride (25·6 m-moles) and 0·45 ml. (25 m-moles) of tritiated water (specific radioactivity 1·33 mc/m-mole), was added and the solution was boiled under reflux for 2 hr. The excess of acetic acid was then recovered by distillation *in vacuo*. The residual oil was heated at 160° at 20 mm. Hg for 0·5 hr. and then boiled under reflux with 5 N-HCl (25 ml.) for 4 hr. The solution was evaporated to dryness, excess of HCl was removed by evaporating twice more after addition of 10 ml. of water, and the residue was dissolved in water (30 ml.). The solution was neutralized to methyl red with dilute aq. NH_3, when *S*-benzylcysteine separated and was filtered off (yield 0·34 g., 54%).

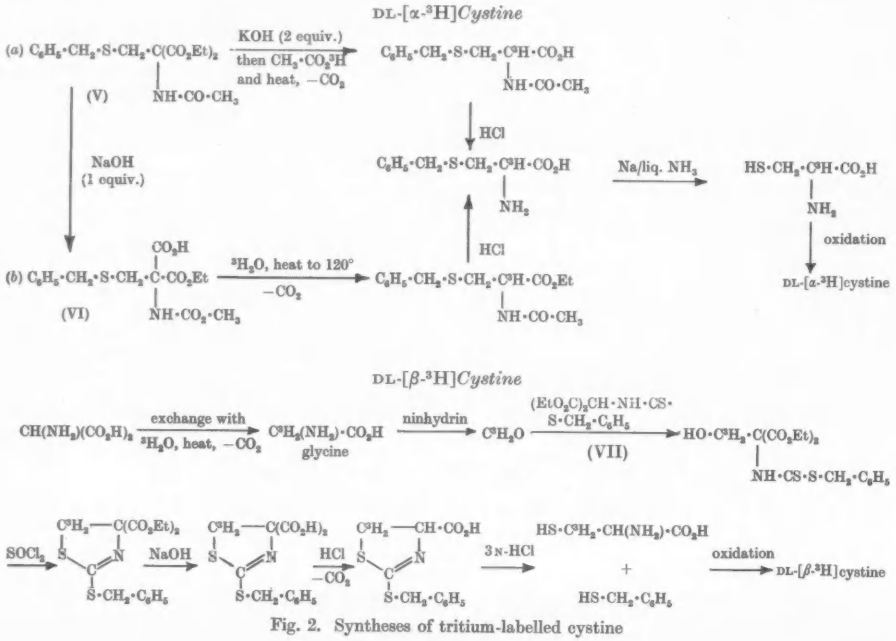


Fig. 2. Syntheses of tritium-labelled cystine

DL-[α-^{3}H]*Cystine*. *S*-Benzyl-[α-^{3}H]cysteine was dissolved in liquid NH_3 and small pellets of Na were added till there was a persistent blue colour. The colour was just discharged with NH_4Cl and the liquid NH_3 was allowed to evaporate. The residue was dissolved in water (30 ml.) and extracted with ether. The aqueous layer was acidified with HCl, and I_2 in ethanol was added until a persistent brown colour appeared. Excess of I_2 was decomposed with $Na_2S_2O_3$ and the solution was neutralized with dilute NH_3, when DL-[α-^{3}H]cystine (50·9 mg.) separated. A second crop (23·1 mg.) was obtained by adding an equal volume of ethanol to the mother liquors. The total yield was 74·0 mg. (37%).

On the assumption that the starting material contains three exchangeable hydrogen atoms (i.e. two carboxyl hydrogens and one hydrogen on the acetamido nitrogen atom), one of which becomes stable to further exchange with non-isotopic water after decarboxylation, the maximum radioactivity of the labelled cystine could be $3·62 \times 10^5$ counts/min. The actual radioactivity of the DL-[α-^{3}H]-cystine was $1·15 \times 10^5$ counts/min., or 32% of the theoretical value.

(*b*) *Ethyl hydrogen α-acetamido-β-benzylthiomethylmalonate* (*VI*). To a solution of the diester (V) (1·757 g., 5 m-moles) in ethanol (10 ml.) N-NaOH (5 ml.) was added. After 18 hr. at room temperature, the solution was evaporated to dryness *in vacuo* (bath temperature <45°). The residue was dissolved in water, extracted with ether and concentrated *in vacuo*. The solution was acidified with 2N-HCl, when a white precipitate was obtained which was filtered off, washed with water and dried. Recrystallization from chloroform by adding light petroleum (b.p. 60–80°) afforded the *half-ester* (VI), m.p. 126–127° with evolution of CO_2 (Found: C, 56·6; H, 6·07; N, 4·63; S, 10·6. $C_{15}H_{19}O_5NS$ requires C, 55·4; H, 5·88; N, 4·31; S, 9·85%). The high figure for C is unlikely to be due to contamination with unchanged starting material, since decarboxylation of the crude product (m.p. 125°) at 130–150° resulted in the evolution of more than 70% of the theoretical amount of CO_2 collected as $BaCO_3$.

S-*Benzyl*-DL-[α-^{3}H]*Cysteine*. The half-ester (VI) (1·63 g., 5 m-moles) was dissolved in dry tetrahydrofuran, 0·09 ml. (5 m-moles, 6·67 mc) of tritiated water was added from a 0·1 ml. blood pipette and the mixture was kept at room temperature overnight. The solvent was removed by distillation *in vacuo* and the residue was decarboxylated by heating for 0·5 hr. at 130° *in vacuo*. Next day the residue had crystallized. 6N-HCl (20 ml.) was added and the mixture was boiled under reflux for 5 hr. The HCl was removed by distillation *in vacuo*, the residue was dissolved in warm water and the solution was neutralized with N-NaOH. Yield of *S*-benzylcysteine, 0·88 g. (83%).

DL-[α-^{3}H]*cystine*. A portion of the *S*-benzyl-DL-[α-^{3}H]-cysteine (0·7 g.) was converted into cystine, as described under (*a*) above. Yield, 0·15 g. (38%). After addition of 100 mg. of carrier DL-cystine, 105 mg. of a second crop of DL-[α-^{3}H]cystine was isolated. This material had 15% of the specific radioactivity of the first crop and the total yield of labelled cystine is therefore 41%. The radioactivity of the main crop was $3·13 \times 10^4$ counts/min., or 33% of the maximum possible activity ($9·4 \times 10^4$ counts/min.).

DL-[β-^{3}H]*Cystine*

[α-^{3}H]*Glycine*. Ethyl aminomalonate hydrochloride (2·1 g., 10 m-moles) was dissolved in tritiated water (1 ml., 55·5 m-moles, 86 mc) and 0·1 ml. of conc. HCl. The solution was boiled under reflux for 2 hr., when decarboxylation occurred. Next day 6N-HCl (20 ml.) was added and the solution was boiled for a further 4 hr. The HCl was removed by evaporation to dryness, water (2×10 ml.) was then added and the evaporation was repeated. The residue was dissolved in ethanol and diethylamine was added till no further precipitation took place. The product was washed with ethanol and acetone; yield 0·92 g. (122%; the high yield is probably due to some contamination with diethylamine hydrochloride). The glycine was assayed for radioactivity after a 1:100 dilution with carrier and the radioactivity of the undiluted material was calculated to be $1·58 \times 10^5$ counts/min. (30% of the maximum possible counting rate).

[^{3}H]*Formaldehyde*. [α-^{3}H]Glycine (0·375 g., 5 m-moles; $1·58 \times 10^5$ counts/min.) was dissolved in 0·25M-KH_2PO_4 (20 ml.) and ninhydrin (2·2 g.) was added. The mixture was distilled almost to dryness, water (10 ml.) was added and the distillation continued till the total volume of the distillate was 25 ml. The formaldehyde content was determined gravimetrically by preparing the dimedon derivative from 0·7 ml. of the distillate. The yield of formaldehyde dimedon was 4·6 mg. (11%). The poor yield of formaldehyde may have been due largely to inadequate distillation.

DL-[β-^{3}H]*Cystine*. Diethyl *N*-benzylthiothiocarbonyl-aminomalonate (VII; Crawhall & Elliott, 1951) (1·70 g., 5 m-moles) was dissolved in pyridine (40 ml.) and the remainder of the solution of [^{3}H]formaldehyde (24·3 ml.) was added. After 18 hr. at room temperature carrier formaldehyde (0·3 ml. of a 40% solution) was added to complete the reaction. After a further 24 hr., the solvent was removed by distillation *in vacuo* and the residue was dried thoroughly in a vacuum desiccator. The product, which crystallized on addition of light petroleum (b.p. 60–80°) was dissolved in dry ether (5 ml.) and freshly distilled thionyl chloride (15 ml.) was added at 0°. After 3 hr. excess of thionyl chloride was removed *in vacuo* at 20° and the residue was dried in a vacuum desiccator over NaOH. The residual oil was dissolved in ethanol (20 ml.) and sat. aqueous NaOH was added till well alkaline (pH about 11). After 45 min., the solution was adjusted to pH 7 with 3N-HCl and evaporated to dryness. After addition of 3N-HCl (40 ml.) the solution was boiled under reflux for 2 hr. and again evaporated to dryness. The residue was dissolved in water (25 ml.) and NaOH was added till the solution was strongly alkaline. After 30 min., the solution was acidified with an excess of conc. HCl, phenylmethanethiol was removed by extracting once with ether and the aqueous phase was boiled under reflux for 18 hr. Excess of HCl was removed by distillation to dryness and the residue was thoroughly extracted with ethanol (3×20 ml.). The insoluble material (NaCl) was removed by centrifuging and the ethanolic solution was treated with I_2 in ethanol till the oxidation was complete (starch being used as external indicator). Diethylamine was added to precipitate DL-[β-^{3}H]cystine, which was centrifuged, washed with ethanol and acetone and dried (yield, 0·157 g., 26%). The radioactivity of this material, assayed after 100-fold dilution of a portion with non-radioactive DL-cystine, was found to be $1·25 \times 10^4$ counts/min. The molar radioactivity of the DL-[β-^{3}H]cystine was thus about 13% of that of the [α-^{3}H]glycine, in agreement with the approx. ninefold dilution of the [^{3}H]formaldehyde with non-isotopic formaldehyde.

RESULTS

Synthesis of ^{3}H*-labelled cystine*

Both [α-^{3}H]cystine and [α-^{3}H]glycine used for synthesizing [β-^{3}H]cystine had only about 30% of the expected specific radioactivity. This result may be due either to incomplete incorporation of tritium during the initial exchange reaction between the malonic esters and labelled water or, more probably, to loss of labelled α-hydrogen atoms during the subsequent acid hydrolysis. Cystine and glycine are known to undergo exchange of hydrogen when heated with 6N-HCl for several days (Rittenberg, Keston, Schoenheimer & Foster, 1938) and even under the less drastic conditions used in the present work (boiling with 5 or 6N-HCl for some hours) partial equilibration of the labelled α-hydrogen atoms with the unlabelled water may have occurred. It seems possible that hydrolysis with more dilute acid or for shorter periods would have reduced this exchange reaction, but this possibility was not investigated since sufficient labelled cystine was obtained.

Oxidative decarboxylation of an α-amino acid to the corresponding aldehyde is known to take place without loss of labelled hydrogen from the α-position (see Spenser, Crawhall & Smyth, 1956) and [^{3}H]formaldehyde was therefore prepared from [α-^{3}H]glycine by this route, which involves only two stages with tritiated water as starting material.

Chemical yields were generally good, but the conversion of labelled glycine into formaldehyde was unsatisfactory. In earlier experiments yields of at least 50–60% were obtained in this reaction, and the poor yield of labelled material may have been due to less efficient steam-distillation.

Utilization of tritium-labelled cystine for penicillin biosynthesis

DL-[α-^{3}H]Cystine was readily utilized for penicillin formation both in complete fermentations and by washed mycelium, although the dilution of isotope was significantly greater than with ^{14}C-labelled cystine (Table 2, Expt. nos. 1*a*, 2, 5 and 6), probably owing to an isotope effect, as discussed below. DL-[β-^{3}H]Cystine also gave rise to labelled penicillin (Table 2, Expt. no. 3). An accurate comparison of the utilization of α- and β-^{3}H-labelled cystine is not possible since the experimental details, particularly the amount of labelled compound added and the times of addition, were different (see Table 1). It has, however, been previously found that the specific radioactivity of penicillin is approximately proportional to the amount of labelled L-cystine added, at least in the range 35–135 mg./l. (Arnstein & Grant, 1954*b*), and the higher isotope dilution in the experiment with DL-[β-^{3}H]cystine (Expt. no. 3) is therefore not unexpected.

As in previous experiments with cystine labelled with ^{14}C, ^{35}S or ^{15}N (Arnstein & Grant, 1954*b*) the molar radioactivity of penicillin derived from [α-^{3}H]cystine was somewhat greater than that of the cysteine in the mycelial protein (Table 3). The radioactivity of the other eight amino acids isolated from the mycelium was insignificant compared with that of cysteine. It is clear, therefore, that re-utilization of isotopic hydrogen, arising from catabolism of the labelled cystine, is accompanied by large dilution of specific radioactivity.

Degradation of the penicillin biosynthesized from tritium-labelled cystine (Table 4) showed that the penicillamine moiety contained less than 10% of the total radioactivity.

Table 2. *Comparison of penicillin biosynthesis from* ^{14}C- *and* ^{3}H*-labelled cystine*

The radioactivity is expressed as μc of ^{14}C/m-mole of substance or, for tritium, as counts/min. × mol.wt. × 10^{-6}. Details of Expts. nos. 1*a* and 3 are given in the Experimental section and in Table 1. In the washed-mycelium expt. 500 ml. conical flasks, each containing 5 g. (wet wt.) of mycelium, 50 ml. of 0·005% potassium phenylacetate, pH 6·9, and 0·25 mg. of labelled cystine, were incubated on a rotary shaker for 4·5 hr. (see Halliday & Arnstein, 1956), two flasks being used for each expt. At the end of the incubation the appropriate duplicate flasks were combined, penicillin was estimated by bioassay and isolated in the usual way after addition of carrier sodium benzylpenicillin (50 mg.).

Expt. no.	Precursor	Amount added (mg./l.)	Molar radioactivity: Cystine added* (*A*)	Molar radioactivity: Penicillin (*B*)	Molar radioactivity: Dilution (*A*/*B*)
1*a*	DL-[α-^{3}H]Cystine	258·6	0·86	0·119	7·2
2†	DL-[β-^{14}C]Cystine	267·5	1·91	0·47	4·1
3	DL-[β-^{3}H]Cystine	52·9	1·51	0·076	19·9
4†	L-[β-^{14}C]Cystine	34·6	2·01	0·145	14·3
		Washed-mycelium expt.			
5	DL-[α-^{3}H]Cystine	5·0	13·7	0·68‡	20·2
6	DL-[β-^{14}C]Cystine	5·0	61·4	6·75‡	9·1

* Calc. as cysteine. † From Arnstein & Grant (1954*b*).
‡ Corrected for dilution by carrier sodium benzylpenicillin.

When DL-[α-^{3}H]cystine was used as a precursor, most of the radioactivity was found in the formaldehyde which arises from $C_{(6)}$ of penicillin (Table 4). The quantitative measurement of the radioactivity of the formaldehyde dimedon was, however, not entirely satisfactory. In degradation no. III a persistently high counting rate was obtained, and the observed radioactivity of the dimedon in degradation no. I was, if anything, lower than that expected. The reason for this variation is unknown, but no change was observed on repeated recrystallization of these samples. The figures for the radioactivity of formaldehyde dimedon must therefore be considered less reliable than those for the other compounds, although the interpretation of these experiments is not affected by the difficulty in assaying this compound.

DISCUSSION

Comparison of the utilization of DL-[β-^{14}C]- and DL-[α-^{3}H]-cystine shows that the latter is utilized with approximately twice the dilution of molar radioactivity as the former. It is unlikely, however, that this difference is due to loss of the isotopic hydrogen by a chemical reaction, such as dehydrogenation to an αβ-unsaturated amino acid derivative. Such a mechanism would result in complete removal of the label; although some indirect incorporation of isotopic hydrogen might occur during subsequent steps in penicillin formation, the radioactivity of the penilloaldehyde moiety [$C_{(5)}+C_{(6)}$ of penicillin] would then be expected to be only of the same order of magnitude as that found in penicillamine or in the mycelial amino acids other than cystine. Moreover, the retention of label during the conversion of [α-^{3}H]cystine into penicillin is also demonstrated by the finding that the specific radioactivity of the penilloaldehyde moiety, which is known to be derived from L-cystine (Arnstein & Grant, 1954*b*), was greater than that of the mycelial cysteine. A similar relationship between the radioactivity of penicillin and that of mycelial cysteine has been observed previously in experiments with ^{14}C-labelled cystine (Arnstein & Grant, 1954*b*).

The less efficient utilization of tritium- compared with carbon-labelled cystine for penicillin biosynthesis is probably due to a quantitatively important 'isotope effect'. It is well known that in chemical or biochemical reactions the carbon–tritium bond is less reactive than the carbon–deuterium or carbon–hydrogen bond, which may cause considerable differences in rates of reactions involving rupture of such bonds. On the other hand, the corresponding differences between the isotopes of carbon are quantitatively much less serious, and important fractionation of isotopes thus occurs mainly with the isotopes of hydrogen (for a recent review see Arnstein & Grant, 1957). Since the α-hydrogen atom of cystine apparently does not undergo a chemical reaction during penicillin biosynthesis, the observed isotope effect must be ascribed to a physical interaction. It seems possible that during synthesis of peptide bonds such a physical interaction between the α-hydrogen atom of an amino

Table 3. *Comparison of radioactivity of mycelial amino acids and penicillin isolated from a fermentation with* DL-[α-^{3}H]*cystine*

The amino acids were isolated from the mycelium obtained in Expt. no. 1*a* (Tables 1 and 2) as described in the Experimental section.

Compound	Radioactivity: Counts/min.	Radioactivity: 10^{-3} (Counts/min. × mol.wt.)
Leucine	1·9	<1·0
Glutamic acid	3·8	<1·0
Threonine	5·7	<1·0
Valine	5·8	<1·0
Alanine	6·0	<1·0
Aspartic acid	8·4	1·1
Serine	9·7	1·0
Glycine	13·0	1·0
Cysteic acid	384·0	65·0
Penicillin	266·7	119·2

Table 4. *Degradation of penicillin biosynthesized from* DL-[α-^{3}H]- *or* DL-[β-^{3}H]-*cystine*

Degradations nos. I and II were carried out with mixed samples of the *N*-ethylpiperidine salt of benzylpenicillin from experiments no. 1*a* and 5 (Table 2), to which carrier had been added. Degradations nos. III and IV were done with the *N*-ethylpiperidine salt from Expts. nos. 1*b* and 3 (Tables 1 and 2) respectively, also after dilution with carrier.

Labelled precursor added to fermentation		DL-[α-^{3}H]Cystine			DL-[β-^{3}H]-Cystine
Degradation no.		I	II	III	IV
Compounds used for tritium assay	Position(s) of H atom(s) in penicillin*	10^{3-} × Molar radioactivity (counts/min. × mol.wt.)			
N-Ethylpiperidine salt of benzylpenicillin	All	20·7	20·9	21·5	17·0
*iso*Propylidenepenicillamine	3, 10, 11	–	–	0·9	1·4
Penilloaldehyde 2:4-dinitrophenylhydrazone	5, 6	22·2	25·1	20·0	15·5
Formaldehyde dimedon	6	16·9	–	27·6	–

* See formula (I). – Signifies compound not isolated.

acid and the enzyme might result in the preferential utilization of molecules which do not contain tritium. Alternatively, uptake of the labelled cystine into the mycelial cells by a mechanism involving interaction with the α-hydrogen atom might similarly lead to discrimination against the labelled molecules. It is noteworthy that the isotope effect observed in the present work on penicillin biosynthesis appears to be quantitatively similar to that which has been found in the biological oxidation of tritium-labelled methanol to formate (Rachele, Kuchinskas, Knoll & Eidinoff, 1954), but further work would be necessary to establish this point conclusively.

The utilization of cystine labelled with tritium in the α-position excludes the participation of an αβ-dehydrocysteine derivative in penicillin biosynthesis. Although the detailed mechanism of β-lactam ring formation is not clear, it seems likely that an oxidative condensation is involved. The observation that one of the β-hydrogen atoms of cystine is incorporated into the penilloaldehyde moiety, presumably at $C_{(5)}$ of penicillin, places certain restrictions on possible mechanisms. Thus oxidation of the β-methylene group of cysteine to a carbonyl group is excluded, since this would involve complete loss of isotopic hydrogen from [β-^{3}H]cystine. On the assumption that cysteinylvaline (VIII) is an intermediate in penicillin formation, the present results are compatible with two possible alternative mechanisms, namely the removal of two hydrogen atoms by dehydrogenation (Fig. 3, *A*), or oxidation of cysteine to the thioaldehyde (X) followed by formation of the C–N bond with loss of a proton from the peptide nitrogen atom (Fig. 3, *B*). At present it is not possible to differentiate between these two mechanisms, but the initial step of pathway *B* is analogous to the conversion of alcohols into aldehydes by alcohol dehydrogenase, which also involves removal of only one of the α-hydrogen atoms of the alcohol (Vennesland & Westheimer, 1954).

Conversion of the intermediate β-thiol-β-lactam (IX) into the thiazolidine–β-lactam ring structure of penicillin may be envisaged as involving αβ-dehydrogenation of the valine moiety to give (XI), followed by addition of the thiol group, as shown in Fig. 3. The final step in penicillin biosynthesis would then consist of the acylation of compound (XII), which has been claimed to be formed in the absence of side-chain precursor (phenylacetic acid) in cultures of *Penicillium chrysogenum* Q 176 (Kato, 1953*a*, *b*).

SUMMARY

1. DL-[α-^{3}H]Cystine and DL-[β-^{3}H]cystine have been synthesized from tritiated water and used to investigate possible mechanisms of penicillin biosynthesis by *Penicillium chrysogenum*.

2. Both α- and β-^{3}H-labelled cystine gave rise to labelled penicillin.

3. Chemical degradation of the penicillin showed that nearly all the isotope was present in the penillo-

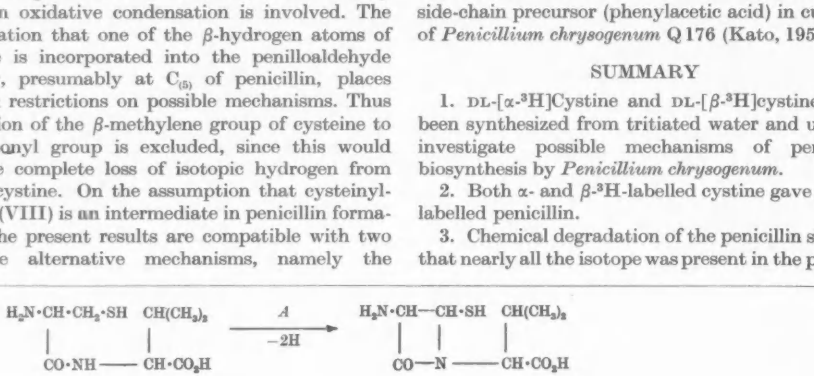


Fig. 3. Suggested pathways of penicillin biosynthesis.

aldehyde moiety, which corresponds to positions 5 and 6 of the molecule. Further degradation indicated that most of the radioactivity of penicillin derived from [α-^{3}H]cystine was located at position 6, as expected from intact incorporation of the labelled cystine.

4. In a fermentation experiment with [α-^{3}H]-cystine the molar radioactivity of penicillin was greater than that of the cysteine in the mycelial protein.

5. These results show that the α-hydrogen atom and one of the β-hydrogen atoms of cystine are retained during penicillin biosynthesis.

6. The significance of these findings is discussed with particular reference to possible mechanisms of β-lactam-ring formation.

We wish to thank Dr J. W. Cornforth, F.R.S., for most helpful discussions concerning the mechanism suggested in this paper for the biosynthesis of the β-lactam ring of penicillin.

REFERENCES

Arnstein, H. R. V. & Clubb, M. E. (1957). *Biochem. J.* **65**, 618.
Arnstein, H. R. V., Clubb, M. & Grant, P. T. (1954). *Proc. 2nd Radioisotope Conf., Oxford*, **1**, 306. London: Butterworths Scientific Publications.
Arnstein, H. R. V. & Crawhall, J. C. (1953). *Biochem. J.* **55**, 280.
Arnstein, H. R. V. & Crawhall, J. C. (1957). *Biochem. J.* **65**, 18P.
Arnstein, H. R. V. & Grant, P. T. (1954*a*). *Biochem. J.* **57**, 353.
Arnstein, H. R. V. & Grant, P. T. (1954*b*). *Biochem. J.* **57**, 360.
Arnstein, H. R. V. & Grant, P. T. (1956). *Bact. Rev.* **20**, 133.
Arnstein, H. R. V. & Grant, P. T. (1957). *Progr. Biophys.* **7**, 165.
Arnstein, H. R. V. & Stanković, V. (1956). *Biochem. J.* **62**, 190.
Banks, T. E., Blow, L. W. & Francis, G. E. (1956). *Biochem. J.* **64**, 408.
Banks, T. E., Crawhall, J. C. & Smyth, D. G. (1956). *Biochem. J.* **64**, 411.
Crawhall, J. C. & Elliott, D. F. (1951). *J. chem. Soc.* p. 2071.
Garrow, J. & Piper, E. A. (1955). *Biochem. J.* **60**, 527.
Halliday, W. H. & Arnstein, H. R. V. (1956). *Biochem. J.* **64**, 380.
Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
Kato, K. (1953*a*). *J. Antibiot.* A, **6**, 130.
Kato, K. (1953*b*). *J. Antibiot.* A, **6**, 184.
Popják, G. (1950). *Biochem. J.* **46**, 560.
Rachele, J. R., Kuchinskas, E. J., Knoll, J. E. & Eidinoff, M. L. (1954). *J. Amer. chem. Soc.* **76**, 4342.
Rittenberg, D., Keston, A. S., Schoenheimer, R. & Foster, G. L. (1938). *J. biol. Chem.* **125**, 1.
Smith, E. Lester & Hockenhull, D. J. D. (1952). *J. appl. Chem.* **2**, 287.
Spencer, I. D., Crawhall, J. C. & Smyth, D. G. (1956). *Chem. & Ind.* p. 796.
Stevens, C. M., Vohra, P. & DeLong, C. W. (1954). *J. biol. Chem.* **211**, 297.
Stevens, C. M., Vohra, P., Moore, J. E. & DeLong, C. W. (1954). *J. biol. Chem.* **210**, 713.
Vennesland, B. & Westheimer, F. H. (1954). In *A Symposium on the Mechanism of Enzyme Action*, p. 357. Ed. by McElroy, W. D. & Glass, B. Baltimore: The John Hopkins Press.
Wood, J. L. & du Vigneaud, V. (1939). *J. biol. Chem.* **131**, 267.

Some Spontaneous Reactions of *OO*-Dimethyl *S*-Ethylthioethyl Phosphorothiolate and Related Compounds in Water and on Storage, and their Effects on the Toxicological Properties of the Compounds

By D. F. HEATH and M. VANDEKAR*
Unit for Research in Toxicology, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey

(*Received* 18 *December* 1956)

Shortly after beginning to study the biochemistry of Metasystox (a mixture of *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate and its thionate isomer) and some related compounds, we discovered that all our samples, tested biochemically, were impure, and that most of them increased in toxicity on storage. We therefore carried out a few preliminary experiments on the whole group of compounds: *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate, Isosystox (the *OO*-diethyl homologue), the thionate isomer (dimethyl ethylthioethyl phosphorothionate) and the sulphoxide and sulphone of the thiolate (*S*-ethylsulphinylethyl- and *S*-ethylsulphonylethyl-*OO*-dimethyl phosphorothiolates). Contrary to expectation, much of the toxicity of all five compounds, as determined by intravenous injection into rats, remained in the water layers after chloroform extraction of their aqueous solutions.

* Present address: Institute for Medical Research, Zagreb, Yugoslavia.

Heating the four dimethyl compounds increased the toxicity of each considerably, again by the formation of compounds inextractable by chloroform. *OO*-Dimethyl *S*-ethylthioethyl phosphorothiolate, its thionate isomer and Isosystox also apparently underwent similar reactions in water at a much faster rate. In some experiments the toxicities were increased 100-fold.

Spontaneous reactions which can increase the toxicities of such widely used insecticides as Isosystox and Metasystox are obviously important. We also wished to provide a firm basis for biochemical work on some of the dimethyl compounds, which presented interesting features (Vandekar & Heath, 1957). We were therefore led to investigate the reactions of the five compounds in detail, with the results described here.

Work with each compound fell naturally into three parts: first, methods of purification were worked out, mainly using solvent extraction, and the purified compounds were tested as rigidly as possible for chemical purity. Secondly, the reactions of these purified compounds were studied both in water and alone, and the toxic derivatives isolated and identified. In the most complicated case, *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate in water, a kinetic analysis of the reactions was undertaken to show that the reactions postulated accounted quantitatively for the products, thus providing evidence that no other reactions leading to toxic products took place. Thirdly, the basic toxic properties—oral and intravenous LD_{50} and I_{50} values—of the compounds and the main toxic derivatives were found, such precautions being taken as the previous findings indicated.

MATERIALS AND METHODS

Compounds. *OO*-Dimethyl *S*-ethylthioethyl phosphorothiolate [$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$], the corresponding sulphoxide and sulphone [*OO*-dimethyl *S*-ethylsulphinylethyl phosphorothiolate, $(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SO{\cdot}Et$, and *OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate, $(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SO_2{\cdot}Et$] and the thionate isomer, *OO*-dimethyl *O*-ethylthioethyl phosphorothionate

$$[(MeO)_2PS{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}SEt]$$

were kindly given to us by Dr G. Schrader, Farbenfabriken Bayer A.-G., Wuppertal-Elberfeld. We also prepared the sulphinyl derivative by the method of Heath & Lane (1952). *OO*-Diethyl *S*-ethylthioethyl phosphorothiolate, $[(EtO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt]$ was given to us by the courtesy of Dr G. S. Hartley, Fisons Pest Control Ltd., Little Chesterford, Essex.

Phosphorus analyses. These were carried out by the method of Fiske & Subbarow (1925). By preliminary oxidation with bromine water before ashing, evaporation losses were avoided (Gardner & Heath, 1953). Results on non-aqueous samples were generally a few per cent low.

Elementary analyses. These were carried out by Drs Weiler and Strauss, 164, Banbury Road, Oxford, and Miss J. Lewis, National Institute for Medical Research, Mill Hill, London, N.W. 7.

Tests of chemical structure. The main test used was successive partitioning (Heath, Lane & Llewellyn, 1952; Heath, Park & Lane, 1955). A solution of the compound in solvent *A* was repeatedly extracted with an equal volume of solvent *B*. The first extract with *B* was then extracted several times with *A*. One solvent always contained water. Each aqueous fraction was analysed for phosphorus. The aim was to extract often enough to remove most of the compound from *A* and from the first extract in *B*. Extractions with one solvent were often followed by further extractions with others. The calculation of results is not affected by the number of solvents used, so that only the case of two solvents is considered in the next paragraph.

For a pure compound, the ratio of the concentrations in the phases *A* and *B* at equilibrium is a constant, the partition coefficient *K*. Thus the concentrations in solvent *A* before and after extraction with *B* are in the ratio $(K+1):K$. Deviations from this relationship indicate impurities. The apparent values of *K*, as determined by phosphorus analysis, may differ a little according as *A* is extracted with *B* or vice versa if volume changes occur during partitioning. With very volatile solvents such changes are difficult to avoid, so that the two sides, *A* extracted with *B*, and *B* with *A*, had occasionally to be treated as two independent series, with some loss of sensitivity. The test is most sensitive if *K* equals 1, so solvents were chosen normally so that *K* was about unity.

The test was used in three ways in this work, and the validity of the results depends in part on its reliability for each purpose. The partition coefficient of a compound between two solvents is a characteristic property. A change in the structure of a compound will nearly always change the partition coefficient by a factor of at least two (see Seidell, 1941; Collander, 1949), except where the change is isomerization without much change in the type of bonding throughout the molecule as, for example, in

$$(MeO)(MeS)PO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$$

and

$$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt,$$

which may have very similar coefficients. Thus the validity of the test for the three ways in which it was used may be summarized as follows:

(1) As a test of purity it was probably very sensitive except to *S*-methyl isomers, of which several per cent may be undetectable.

(2) As a check that a compound is unchanged by a given treatment, for example, heating or storage, the test was reliable unless isomerization to *S*-methyl isomers took place, or, very improbably, two or more successive reactions led to a product with the coefficient of the parent compound.

(3) As a method for identifying the products of reactions, the test indicated only the probable nature of the product. By suitable design the test separated products fairly completely from the parent compounds, and gave their partition coefficients. Agreement between these coefficients and those of known compounds likely to be found suggested that these products were identical with the known compounds. There may, however, be other compounds with the same coefficient which can be produced.

The rate at which cholinesterase recovered its activity, after being inhibited by a short period of contact with a

compound, was also used to test structure. The method is due to Aldridge (1953), who showed that the rate of recovery was dependent on the structure of that part of the inhibitor which remained on the enzyme. Thus in esters, RR'PO·X, where R and R' are alkoxy or similar groups and X is a replaceable acidic radical, the rate of recovery depends upon R and R'.

The method of Aldridge (1953) was used, unchanged, except that the inhibited enzyme was washed three times to remove excess of inhibitor.

Electrophoretic separations. These were carried out at 4°, in the apparatus described by Flynn & Mayo (1951), on strips of Whatman 3MM filter paper, 36 cm. × 5·5 cm., at 550 v in an electrolyte of 0·35% sodium acetate trihydrate and 0·16% glacial acetic acid in water, pH 5·3. For analysis the strips were cut up and eluted by immersion in water for at least 30 min.

Anticholinesterase activities. These were determined with sheep-erythrocyte cholinesterase for 30 min. at 37°, followed by the determination of the cholinesterase activity remaining (Aldridge, 1950). The rates of CO_2 evolution were calculated by the method of Aldridge, Berry & Davies (1949). I_{50} values were determined by choosing at least four concentrations which produced 40–60% inhibition. For the dimethyl phosphate esters the graph of log (percentage activity) against concentration of inhibitor was not a straight line because the inhibition, as with all dimethyl phosphate esters, is reversible. It is, however, nearly straight over the short range 40–60%. The average gradient of the best straight line over this range does not pass through 2, i.e. log (100%) at zero concentration. From over a hundred curves the best line appeared to pass through 1·95. The line chosen was therefore that which passed through this point and gave the best fit to the experimental points, and the I_{50} was taken as the concentration on this line corresponding to 50% inhibition.

I_{50} values of purified compounds were determined by using solutions containing concentrations of inhibitor found by phosphorus assay. This enabled us to use freshly purified compounds with the minimum of delay.

Concentration of inhibitor in a sample. This was usually expressed as the number of times the sample had to be diluted to give an I_{50} concentration, and was called the I_{50} dilution.

Preparation of solutions for toxicity and anticholinesterase tests. Purified compounds were stored at −30°. Solutions in water were prepared immediately before testing. Solutions in non-aqueous solvents were concentrated *in vacuo*, water was added and the evacuation continued until the non-aqueous solvent had disappeared. Non-aqueous solvents dissolved in aqueous solutions were blown out with air. The aqueous solutions were either tested immediately, or stored at −30°.

Toxicity tests. These were carried out on male and female albino rats of the same stock. Intravenous injections were made by the tail vein. Surviving animals were observed until all symptoms disappeared and for at least 24 hr. Rough LD_{50} values were obtained on 4–6 animals. Those given in Table 11 were obtained on sixteen animals, estimated by the method of Thompson & Weil (1952), the tables for median-effective dose given by Weil (1952) being used. The concentrations of active compounds in the solutions used for test were determined by phosphorus assay.

Constitution and purification of samples

Our samples had been distilled, a process which can lead to complex breakdown of dimethyl phosphate esters (Macpherson & Johnson, 1956). Elementary analyses were carried out to show that gross impurities were absent. The samples were then subjected to successive partitioning, purified where necessary, and the rate of recovery of cholinesterase inhibited by them was determined.

OO-*Dimethyl* S-*ethylthioethyl phosphorothiolate.* Found: C, 31·4; H, 6·4; S, 29·7, 24·9; P, 13·1, 12·4, 13·1%. $C_6H_{15}O_3S_2P$ requires C, 31·3; H, 6·52; S, 27·8; P, 13·5%. As there is a general tendency for sulphur analysis to be somewhat inaccurate in compounds with P–S bonding and for phosphorus to be found a little low in fairly volatile compounds such as these, the results indicated that the sample consisted originally of the stated compound or its isomers.

Successive partitioning (Table 1) showed mainly one compound, of partition coefficient (light petroleum–water) $1{\cdot}60_5$, contaminated with 0·60% of compounds not extractable from water by light petroleum or $CHCl_3$. The compound was freed from water-soluble impurities by extracting a $CHCl_3$ solution with water.

Cholinesterase, inhibited *in vitro* with the purified compound, recovered its activity with a half-life of 1·3 hr., the characteristic half-life for cholinesterase inhibited with a dimethyl phosphate ester.

OO-*Dimethyl* O-*ethylthioethyl phosphorothionate.* Found: C, 31·0; H, 6·66; S, 29·3, 27·2; P, 12·0, 12·65, 13·3%. $C_6H_{15}O_3S_2P$ requires C, 31·3; H, 6·52; S, 27·8; P, 13·5%, in satisfactory agreement.

Successive partitioning revealed that 36·2% of the sample had the light petroleum–water partition coefficient of the thiolate isomer, presumably formed by isomerization

Table 1. *Successive partitioning of* OO-*dimethyl* S-*ethylthioethyl phosphorothiolate*

Numerals in parentheses refer to the serial numbers in the table. A solution of the compound in water (1) was extracted four times with light petroleum, b.p. 100–120°, and the water layers were assayed for phosphorus after each extraction (2–5). (5) was then extracted with $CHCl_3$, leaving (6). The first light-petroleum extract was extracted five times with water, and each water extract assayed (7–11). The calculated results were obtained assuming that (1) contained 324 μg. of P/ml., that K (light petroleum–water) was 1·605, and that the fraction not extracted by $CHCl_3$ could not be extracted by light petroleum.

No.	Concn. of phosphorus in aqueous layers (μg./ml.) Found	Calc.
1	330, 325	324
2	125, 126	126
3	49·3, 49·9	49·4
4	19·9, 18·7	20·2
5	8·53, 8·71	8·96
6	1·96, 1·96	—
7	77·5, 76·9	76·2
8	46·0, 45·1	46·9
9	29·0, 29·0	29·0
10	17·5, 17·8	17·8
11	11·0, 11·0	11·0

Table 2. *Successive partitioning of purified* OO-*dimethyl* O-*ethylthioethyl phosphorothionate*

Light petroleum, b.p. 100–120°, and 20% (v/v) aqueous methanol were equilibrated at 4°. The thionate was added to aqueous methanol (1) and rapidly extracted six times with an equal volume of light petroleum, leaving six methanol layers for assay (2–7). The first light-petroleum extract was rapidly extracted four times with aqueous methanol (8–11). The whole process took 20 min. The results were calculated on the assumption that (1) contained 123·0 μg. of P/ml., of which 0·149 μg. of P/ml. could not be extracted, and that K (light petroleum–methanol) was 1·70 when methanol was extracted by light petroleum, but was 1·94 when light petroleum was extracted by methanol. The difference was due to carry-over in this rapid experiment, when to avoid decomposition of thionate time could not be spent in cleaning funnels between extractions.

	Concn. of phosphorus in methanol layers (μg./ml.)	
No.	Found	Calc.
1	127	123
2	45·0	45·3
3	17·0	16·9
4	6·33	6·35
5	2·46	2·44
6	0·92	1·01
7	0·50	0·46
8	28·4	29·8
9	20·3	19·7
10	13·0	13·0
11	8·86	8·45

on ageing (Henglein & Schrader, 1955). The thionate is more oil-soluble than the thiolate, and was therefore separated as follows: crude thionate (20 g.) was dissolved in light petroleum, b.p. 100–120° (400 ml.), and extracted rapidly three times with successive 400 ml. portions of 20% (v/v) aqueous methanol. The light-petroleum layer was evaporated rapidly *in vacuo* finally at 50° under 0·1 mm. Hg. The product is likely to contain some solvent. Phosphorus assay gave the thionate content as 95·7%. Successive partitioning of the product at 4° revealed no impurities (Table 2).

The compound reacted too rapidly with water to test the recovery of cholinesterase inhibited by it.

OO-*Dimethyl* S-*ethylsulphinylethyl phosphorothiolate.* A sample was prepared by the method of Heath & Lane (1952), and purified as follows: the product (5 g.) in water (50 ml.) was extracted with $CHCl_3$ (3 × 50 ml.) to separate it from H_2O_2. From the $CHCl_3$ extract a water solution was prepared. The impurities most likely to be present are the sulphide and sulphone. The aqueous solution was therefore extracted with three equal volumes of 1:2-dichloropropane, which removes all the sulphide and about 95% of the sulphone. The sulphoxide was then extracted with $CHCl_3$ and concentrated, finally at 60° under 0·1 mm. Hg. Phosphorus assay gave 93% of sulphoxide, the remainder being presumably $CHCl_3$.

Successive partitioning showed the compound to be substantially pure (Table 3). It contained no appreciable amount of material extractable by light petroleum, as shown by the low and equal concentrations of phosphorus found in the light petroleum fractions A and B. Samples 14–16, Table 3, show that some compound was present which had a partition coefficient near that of the sulphone. We have assumed that this was sulphone, because it was a likely impurity. The quantity estimated is very sensitive to the partition coefficient taken, so that it can be estimated only as 0·8±2%. The remainder, except traces of inextractable compounds (0·036%), was sulphoxide.

Table 3. *Successive partitioning of* OO-*dimethyl* S-*ethylsulphinylethyl phosphorothiolate*

A solution in water (1) was extracted with light petroleum, b.p. 100–120°, twice, yielding light-petroleum fractions A and B. The aqueous residue was then extracted with benzene, giving a water layer (2) and a benzene layer (C). The aqueous residue was then extracted twice with redistilled 1:2-dichloropropane, giving D and E and leaving an aqueous layer (3). This was extracted five times with methylene dichloride and twice with $CHCl_3$, leaving seven aqueous layers (4–10). The dichloropropane layers were bulked, extracted once with water (water layer 11) and concentrated 15·2-fold. The concentrate was then extracted four times with water (water layers 12–15), leaving a dichloropropane layer (16). The first methylene dichloride extract was extracted five times with water, giving water layers (17–21). The results were best fitted by assuming that the sample contained 99·16% of sulphoxide, 0·806% of sulphone and 0·036% of compounds inextractable by any solvent. Partition coefficients used in the calculation are given at the bottom of the table. The starred values were calculated from the results, the remainder determined independently.

	Phosphorus (μg./ml.)	
No.	Found	Calc.
1	357, 356	358
2	345, 342	351
3	286, 285	291
4	108, 110	108
5	40·8, 40·8	40·3
6	14·0, 15·3	15·0
7	5·72, 5·68	5·67
8	2·20, 2·13	2·19
9	0·51, 0·62	0·51
10	0·18, 0·19	0·20
11	26·8, 27·2	26·9
12	36·6, 36·6	38·3
13	5·03, 4·96	4·99
14	1·54, 1·47	1·47
15	0·79, 0·78	0·78
16	1·16	1·29
17	66·2, 64·8	68·0
18	42·2, 42·1	42·7
19	27·0, 26·4	26·8
20	16·9, 16·9	16·9
21	10·9, 10·6	10·6
A	0·021, 0·012	0·014
B	0·021, 0·012	0·014
C	7·58, 7·35	7·47
$D+E$	27·5, 29·2	30·0

	Partition coefficients			
	Light petroleum–water	Benzene–water	Dichloropropane–water	Methylene dichloride–water
Sulphoxide	0·00001*	0·0189*	0·0955*	1·692*
Sulphone	0·001	0·36	1·70	∞

Cholinesterase, inhibited with the sample, recovered its activity with a half-life of 1·3 hr.

OO-*Dimethyl* S-*ethylsulphonylethyl phosphorothiolate*. The crystalline sample given us contained about 2% of compounds not extractable by $CHCl_3$. To remove the *S*-ethylthioethyl compound, an aqueous solution, 5 g. in 100 ml., was extracted four times with light petroleum. The sulphone was then separated from the sulphoxide by extracting the aqueous solution twice with 100 ml. of 1:2-dichloropropane. The bulked extracts were washed with 40 ml. of water and concentrated *in vacuo*, finally at 50° under 0·1 mm. Hg. Phosphorus assay gave 93·8% of sulphone.

Successive partitioning showed only traces of any impurity (Table 4). The discrepancies between the found and calculated phosphorus concentration in samples 9 and 10, Table 4, together with the low $CHCl_3$–water partition coefficient, which, determined independently, is about 30, may indicate about 0·2% of sulphoxide.

Cholinesterase inhibited with the compound recovered its activity with a half-life of 1·3 hr.

OO-*Diethyl* S-*ethylthioethyl phosphorothiolate*. The sample was originally part of the highly purified compound used by Gardner & Heath (1953). Four years later, 17% was inextractable by $CHCl_3$ from water. To purify it, it was dissolved in light petroleum, b.p. < 40°, washed twice with water and concentrated *in vacuo*, finally at 50° and 0·1 mm. Hg. The purity was tested by using in turn reversed-phase partition chromatography and a simple form of successive partitioning. The chromatographic methods used were communicated privately by Dr I. K. H. Otter, Fisons Pest Control Ltd., Chesterford Park Research Station, Little Chesterford, Essex.

Spots of the sample in *cyclo*hexane were placed on strips of Whatman no. 1 filter paper impregnated with 5% of silicone MS 550 (Hopkin and Williams Ltd.), dried, and equilibrated with moving phase at 25°. In one run the moving phase was 100 ml. of acetone + 15 ml. of water, in the other 100 ml. of ethanol + 15 ml. of water. Both were run by the descending-phase method and developed by Otter's (1955) method. Both gave only one spot, R_F 0·93 and 0·90 respectively. This showed that the sample contained no compounds more oil-soluble than the stated compound.

The compound was dissolved in light petroleum, b.p. < 40°, and extracted with water twice, giving two partition coefficients, $8{\cdot}0_4$ and $8{\cdot}0_4$. The first aqueous extract was back-extracted with light petroleum, giving *K* 8·30. This showed that any impurity present to more than 1% must have a partition coefficient very near that of the pure compound.

Cholinesterase, inhibited with the compound, recovered its activity very slowly, as expected when a diethyl phosphate inhibitor is used.

Partition coefficients. These are summarized for convenience in Table 5. All five compounds can be extracted from water practically completely by three successive extractions with $CHCl_3$.

Table 4. *Successive partitioning of* OO-*dimethyl* S-*ethylsulphonylethyl phosphorothiolate*

An aqueous solution (1) was extracted three times with light petroleum, b.p. 100–120°, giving three light-petroleum extracts (2–4) and leaving an aqueous extract (5). This was extracted five times with 1:2-dichloropropane and twice with $CHCl_3$, leaving aqueous extracts 6–12. The first dichloropropane extract was extracted five times with water, yielding 5 aqueous extracts, 13–17. Theoretical values are calculated on 99·976% of a compound with *K* (light petroleum–water) = 0·0010, *K* (1:2-dichloropropane–water) = 1·70, *K* ($CHCl_3$–water) = 15·7, and 0·024% of compounds inextractable by any solvent.

No.	Phosphorus (μg./ml.) Found	Calc.
1	234	233
2	0·26	0·23
3	0·24	0·23
4	0·24	0·23
5	231, 234	232
6	86·0, 87·0	86·1
7	31·3, 33·3	31·9
8	12·2, 11·7	11·9
9	4·27, 4·26	4·42
10	1·64, 1·62	1·67
11	0·159	0·159
12	0·056	0·056
13	54·3, 56·7	54·2
14	33·2, 34·4	34·1
15	20·8, 21·5	21·5
16	13·3, 13·5	13·5
17	8·0, 8·2	8·5

Table 5. *Partition coefficients of the compounds*

Partition coefficients were determined at room temperature (18–25°) except for those asterisked, which were determined at 4°.

Solvent system	$(EtO)_2PO$[1]	PO[2]	PS[3]	SO[4]	SO_2[5]
Light petroleum (b.p. 100–120°)–20% aq. methanol	~0·8	~0·07*	1·70*	—	—
Light petroleum (b.p. 100–120°)–water	~15	1·61 1·52*	> 20	~0·00001	~0·0010
Benzene–water	V. large	~20	V. large	0·019	0·36
1:2-Dichloropropane–water	V. large	V. large	V. large	0·096	1·70
Methylene dichloride–water	V. large	V. large	V. large	1·69	18·7
$CHCl_3$–water	V. large	V. large	V. large	4·4	~30

[1] $(EtO)_2PO$ = *OO*-diethyl *S*-ethylthioethyl phosphorothiolate.
[2] PO = *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate.
[3] PS = *OO*-dimethyl *O*-ethylthioethyl phosphorothionate.
[4] SO = *OO*-dimethyl *S*-ethylsulphinylethyl phosphorothiolate.
[5] SO_2 = *OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate.

RESULTS

Reactions of the purified compounds

It was found that, when purified as already described, all but the sulphone gave toxic derivatives on heating, storage or in water. The compounds are now dealt with in turn.

OO-*Dimethyl and* OO-*diethyl* S-*ethylthioethyl phosphorothiolates*

As these compounds produced toxic derivatives most rapidly, and conveniently, in water, most attention was paid to aqueous solutions. The toxicity of a freshly prepared 1 % solution of the dimethyl compound increased greatly, the intravenous LD_5 decreasing from 60 to 2 mg./kg. in one day at 35°. All this increase in toxicity was due to the formation of compounds not extractable by chloroform. About 7 % of the compound had decomposed in this time.

To prove that the toxic derivative was not produced by an impurity, e.g. the *S*-methyl isomer, we proved that throughout the decomposition of the compound in water the derivative was produced at the same rate from the same concentration. Table 6 shows that the LD_{50} of the aqueous layer obtained by storing an approximately 1 % solution one day is substantially constant up to 80 % decomposition. The slight upward drift in LD_{50} as the experiment proceeded is explained below. An impurity could produce such results only if it possessed both the same solubility properties and the same rate of reaction with water. Qualitatively similar results were obtained with the diethyl compound, though the reaction was about ten times as slow.

We then showed that the rate of formation of toxic derivatives depends on the square of the concentration of the dimethyl compound, using anticholinesterase activity of samples of solution, freed from parent compound by extraction with chloroform, as a measure of their concentration. Table 7 shows that on diluting the parent compound tenfold the rate of production of inhibitor was decreased 100-fold. The phosphorus analyses showed that at any given time the percentage of the total phosphorus in the water layer was similar at both concentrations. Thus most of the phosphorus in the water layer was in non-toxic compounds and the toxic product was formed by a relatively minor side reaction.

The bimolecularity of the reaction explained the upward drift in LD_{50} in Table 6, as the concentrations of the parent compound drifted downwards during the experiment.

Separation and identification of the toxic derivative of the dimethyl compound. As the inhibitor could not be extracted from water by immiscible solvents we concluded that it was probably ionic, and therefore

Table 6. *Production of toxic derivatives from* OO-*dimethyl* S-*ethylthioethyl phosphorothiolate in water*

A 1 % solution of dimethyl thiolate was stored for one day at 35°. The dimethyl compound was extracted with $CHCl_3$ and the LD_{50} of the aqueous layer determined. A 1 % aqueous solution was prepared from the $CHCl_3$ extract and its LD_{50} found, and the solution stored for several days at 35°. It was then re-extracted, the percentage decomposed determined by phosphorus assay of the aqueous layer, and a 1 % aqueous solution prepared from the $CHCl_3$ layer. This was again stored for one day and re-extracted, and the LD_{50} of the aqueous layer found.

Days from start of decomposition	1	8	19	30
Concn. of compound at start of day (mg./ml.)	9·7	6·7	8·9	6·0
LD_{50} of aqueous layer (intravenous) (mg./kg.)*	0·15–0·22	0·18–0·24	0·27–0·34	0·29–0·46
LD_{50} of $CHCl_3$ layer (intravenous) (mg./kg.)	48	—	35	—
Total decomposition	7·8	43	71	81

* Estimated as the parent compound.

Table 7. *Production of anticholinesterase from* OO-*dimethyl* S-*ethylthioethyl phosphorothiolate in* 0·0001 N-HCl *at* 37°

To remove any toxic derivatives produced on storage, a benzene solution was extracted twice with water and a solution of about 5 mg./ml. in 10^{-4} N-HCl prepared rapidly from it. Some was diluted tenfold with acid of the same concentration and both solutions held at 37° in a thermostat. Samples were taken at set times and extracted three times with $CHCl_3$, and the I_{50} dilutions and phosphorus content of the aqueous layers found. The values quoted are I_{50} dilutions; the errors are about ±10 %.

Initial concn. (mg./ml.)	Concn. of anticholinesterase: Time (hr.) 2	4·5	6	8	25	48	72	146	312	648
5·08	4600	6600	7600	8500	11 300	13 200	16 800	20 100	19 000	8800
0·508	28	75	66	80	93	120	196	227	227	—

tried to isolate it by electrophoresis. The results are shown in Fig. 1.

The inhibitor appeared in a position indicating that it was positively charged; the displacement of the peak towards the cathode was more than two-thirds of the displacement of phosphate ion at pH 5·3 towards the anode, and the profile of the inhibitor and a phosphorus band were very close. Other phosphorus bands appeared in the neutral position and, as expected for hydrolysis products, towards the anode. The spread of the inhibitor band was due to overloading. Experiments with smaller quantities of material gave a well-defined inhibitor band only about 3 cm. wide, but the phosphorus content of such bands was too small for accurate analysis.

Cholinesterase inhibited by the compound recovered its activity with a half-life of 1·3 hr., showing that the compound was a dimethyl phosphate.

These results indicated that the compound was *OO*-dimethyl *S*-ethylsulphonioethylmethyl phosphorothiolate, formed by the reaction:

$$2(MeO)_2PO\cdot S\cdot CH_2\cdot CH_2\cdot SEt \rightarrow MeO\cdot PO_2^-\cdot S\cdot CH_2\cdot CH_2\cdot SEt + (MeO)_2PO\cdot S\cdot CH_2\cdot CH_2\cdot \overset{+}{S}EtMe \quad (1)$$

To confirm this the apparently identical compound was synthesized. Electrophoresis yielded a positively charged compound in 9 % yield with the same I_{50} and hydrolysis rate (Table 8), and moving the same distance in the electrophoretic field.

The isomeric structure,

$$(MeO)_2PO\cdot \overset{+}{S}Me\cdot CH_2\cdot CH_2\cdot SEt,$$

would also be consistent with most of the results, but is unlikely both for chemical and biochemical reasons. The sulphur atom attached to the phosphorus appears to be generally unreactive; oxidation of the *OO*-diethyl sulphide with hydrogen peroxide can give nearly quantitative yields of sulphoxide in which only the other sulphur is attacked (Heath *et al.* 1955). Biochemically the compound is a much more powerful inhibitor than the parent compound (see Table 11), which could reasonably be explained by the similarity between the groups

$$PO\cdot S\cdot CH_2\cdot CH_2\cdot \overset{+}{S}MeEt,$$

and

$$CO\cdot O\cdot CH_2\cdot CH_2\cdot \overset{+}{N}Me_3$$

in acetylcholine. Dr H. S. Hopf (private communication) has shown also that conversion of compounds containing the group $PO\cdot S\cdot CH_2\cdot CH_2\cdot NR_2$ into those containing the group $PO\cdot S\cdot CH_2\cdot CH_2\cdot \overset{+}{N}R_3$ in-

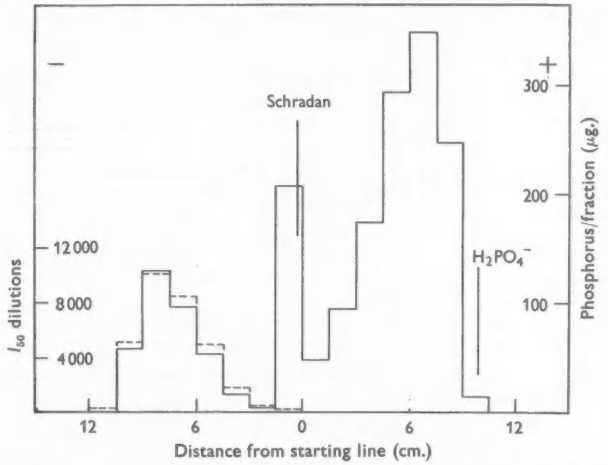


Fig. 1. Electrophoresis of the ionic products from the action of water on *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate at room temperature for 14 days. A 1 % solution of the thiolate in 0·005 N-HCl was left for 14 days at room temperature (about 18°) and then extracted with $CHCl_3$. Three electrophoresis papers (Whatman 3 MM, 5·5 cm. × 36 cm.) were each treated with 0·25 ml. of the aqueous residue at the starting lines, and electrophoresis was carried out with acetate buffer, pH 5·3, at 500 v for 135 min. The papers were cut up into strips 1·5 cm. wide, starting at the starting lines. Equivalent strips from all three papers were bulked, left in 10 ml. of water for 1 hr. and then removed. The aqueous fractions were analysed, for phosphorus and anticholinesterase activity: phosphorus, ——; I_{50} dilutions, - - - -. A fourth paper strip, treated on the starting line with a few micrograms of schradan and KH_2PO_4, was run simultaneously, and developed by the methods already described. The centres of the bands are marked by vertical lines. At pH 5·3 phosphate ion exists almost completely as $H_2PO_4^-$.

creases considerably the activity against the cholinesterase of the locust central nervous system.

The toxic derivative from stored samples. About 4 % of a similar compound with very similar toxicological and charge properties was separated from a stored sample of the dimethyl compound. The intravenous LD_{50} of this sample was about 1 mg./kg.

The toxic derivative from the diethyl compound. Electrophoresis of a stored sample gave a positively charged inhibitor. The ethylsulphonium derivative was synthesized from ethyl iodide as before, and showed the exceedingly toxic properties given in Table 11.

The methylsulphonium derivative of Isosystox was also synthesized and separated. This has already been examined by Fukuto, Metcalf, March & Maxon (1955*b*), but they found an intraperitoneal toxicity to mice of only 1–5 mg./kg., whereas we found the intraperitoneal toxicity to mice to be 0·10 mg./kg. This suggests that their product was probably very impure. Their method of separation would not isolate the compound from by-products, which are formed in quantity.

OO-*Dimethyl* O-*ethylthioethyl phosphorothionate*

Action of water. Henglein & Schrader (1955) showed that this compound isomerized to the *S*-ethylthioethyl isomer in water. The lowest concentration they used was 1 %. We wished to study true solutions (< 0·2 %). We therefore checked that the same reaction took place at such dilutions.

A solution, 0·77 mg./ml. in 9 % (v/v) aqueous ethanol, was stored for 24 hr. at 37° and extracted with chloroform. Successive partitioning showed that the only chloroform-extractable compound had a partition coefficient [light petroleum (b.p. 100–120°)–water] of $1{\cdot}6 \pm 0{\cdot}1$ in agreement with that of the thiolate. The yield was, however, only 42 %. Thus isomerization takes place, but with lower yields than at higher concentrations.

The intravenous toxicity of a solution of concentration 2 mg./ml. increased in 1 hr. at 37° from 220 to 2 mg./kg. This could not be explained either by the formation of thiolate (LD_{50} 64 mg./kg., Table 11) or by the subsequent action of thiolate with itself to form the methylsulphonium derivative, which was a relatively slow reaction at this dilution. Thus the increase in toxicity must have arisen from reactions involving the thionate. An experiment designed to provide more evidence for this is described in Table 9.

Table 9 shows that a solution of thiolate alone produced very little inhibitor, as we expected at this low concentration. However, solutions containing thionate produced inhibitor rapidly, and this inhibitor was nearly completely decomposed by

Table 8. *Properties of* OO-*dimethyl* S-*ethylsulphonioethylmethyl phosphorothiolate prepared in two ways*

The compound was obtained by the action of water on *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate, as described (see Fig. 1) and by the action of methyl iodide (0·1 ml.) on the same compound (0·1 ml.) in ethanol (1 ml.) for 39 hr. at 37°. Hydrolysis rates were calculated from I_{50} dilutions determined on samples taken at various intervals over several weeks from stock solutions. Errors are standard errors.

	Compound prepared by the action on thiolate of	
	Water	Methyl iodide
I_{50}	$3{\cdot}75 \times 10^{-8}$ M	$3{\cdot}95 \times 10^{-8}$ M
$10^5 k_w$ (min.$^{-1}$)	$4{\cdot}05 \pm 0{\cdot}44$ in 0·01 N-HCl	$3{\cdot}66 \pm 0{\cdot}11$ in 0·01 N-HCl $3{\cdot}78 \pm 0{\cdot}27$ in 0·001 N-HCl

Table 9. *Reactions of* OO-*dimethyl* O-*ethylthioethyl phosphorothionate in* 0·0001 N-HCl *with and without admixture of the thiolate isomer, compared with the reactions of thiolate at* 37°

Three solutions were prepared, containing respectively 0·215 mg. of thiolate/ml., 0·397 mg. of thionate/ml., and 0·215 mg. of thiolate + 0·397 mg. of thionate/ml., all in 9 % (v/v) aqueous ethanol, and stored at 37°. Samples taken at various times were extracted with $CHCl_3$, and the aqueous layers assayed for phosphorus and anticholinesterase activity. The aqueous layers were then stored a further day at 37°, and the I_{50} dilutions redetermined.

	Thionate only (0·397 mg./ml.)			Thionate + thiolate (0·397 + 0·215 mg./ml.)			Thiolate only (0·215 mg./ml.)		
Time (min.)	P in aq. phase (%)	I_{50} dilutions 0 days	1 day	P in aq. phase (%)*	I_{50} dilutions 0 days	1 day	P in aq. phase (%)	I_{50} dilutions 0 days	1 day
0	0	1	—	0	1	—	0	1	—
10	42	136	5	41	2370	3	—	5	—
100	67	399	5	68	2500	5	—	—	—
1300	71	9	4	70	61	22	7	17	—

* The percentages in this column are estimated on the thionate alone.

storing for 1 day at 37°. Thus the inhibitor produced from the thionate was not the methylsulphonium derivative of the thiolate, which was considerably more stable than this (see Table 8). Its formation was greatly accelerated by thiolate, indicating that it was formed by a reaction between the thiolate and thionate. This hypothesis accounts also for the finding that, when thiolate was added, most of the inhibitor was formed in the first 10 min., whereas, when thiolate was not added, only one-third of the inhibitor was produced in this time. The rate of formation of inhibitor was probably proportional to the product of the concentrations of thiolate and thionate. Consequently, with thiolate added, the maximum rate would be attained immediately, but in the absence of additional thiolate would only be attained when the product of the concentrations reached a maximum, i.e. when the decomposition of thionate was about half complete. The maximum rate was thus only attained after about 10 min.; for at this time ionic products accounted for 41 % of the thionate phosphorus in both experiments, which, as isomerization proceeded simultaneously, indicated that about half of the thionate had been decomposed by both reactions.

In a further experiment with thionate alone it was shown that the final concentration of inhibitor was roughly proportional to the square of the thionate concentration, which agrees with the hypothesis given above.

Electrophoresis of a sample of the inhibitor from thionate alone showed that it was positively charged. The recovery was 72 %. No phosphorus could be detected in the band, from which it could be shown that the I_{50} was less than 10^{-6} M. The first-order constant for the decomposition of the inhibitor in the presence of other products of the reactions was $7 \pm 1 \times 10^{-3}$ min.$^{-1}$ at 37°.

The structure of the unstable inhibitor is considered in the Discussion. Whatever the reaction yielding it, it was a trivial side reaction, for the quantity of chloroform-inextractable phosphorus produced was the same whether thiolate was added or not (Table 9), although eight times as much inhibitor was formed in the first instance as in the second. This observation, and the figures in Table 9, show that the I_{50} of the unstable inhibitor must be less than 10^{-8} M, i.e. the inhibitor was very powerful.

Kinetics of the decomposition of OO-*dimethyl* S-*ethylthioethyl phosphorothiolate in water*

It has been shown that *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate underwent simultaneous hydrolysis and self-methylation in water. The experiments here showed that another reaction sequence also took place and also affected the toxicity of aqueous solutions. All reactions were carried out in weakly acid solutions, as dialkyl phosphate esters are less sensitive to acids than to bases.

A solution of thiolate in 0·005 N-HCl (5 mg./ml.) was held at 37° for 2 hr. and extracted with chloroform, and the inhibitor in the aqueous layer determined. The I_{50} dilution was 3800. The inhibitor solution was left for 17 hr. at 37°, and the concentration redetermined. The I_{50} dilution was only 370, i.e. 90 % had decomposed in 17 hr.

Now the only inhibitor found in a thiolate solution left for 14 days was the sulphoniothiolate of half-life 13·2 days (see Table 8). Therefore a less stable inhibitor was produced alongside the sulphoniothiolate in the early stages of the reaction. To eliminate the possibility that an unstable impurity produced this inhibitor the experiment was repeated with thiolate that had been pretreated with water for one day at 37° and then purified by extration with chloroform. The results were the same; thus the unstable inhibitor was produced from the thiolate.

These observations could be explained if the thiolate was in dynamic equilibrium with thionate, with which, according to the results of the preceding section, it reacted to form the unstable inhibitor. As the two inhibitors differed considerably in their stability in water, mixtures could be analysed by determining the activity immediately, and after one day's storage. The first gave the total inhibitor, the second the stable inhibitor less the small, calculable proportion decomposed in one day. This was the basis of the following experiments, which were designed to check whether the reactions already postulated were adequate to account for the results observed when allowance was made for the formation of unstable inhibitor.

If the simplest theory is applicable, the rate of decomposition of thiolate is given by

$$-\mathrm{d}A/\mathrm{d}t = k_1 A + k_2 A^2, \qquad (2)$$

where A is the concentration of thiolate. The first term is for the first-order hydrolysis, the second for the second-order production of inhibitors. First, this equation was checked experimentally, by using thiolate pretreated for 1 day at 37° with dilute acid.

In very dilute solution $k_2 A^2$ can be ignored. The rate of hydrolysis was determined by Heath's (1956) method, which is based on the assumption that ionic products cannot be extracted from water by chloroform. In 0·094 % solution in 0·005 N-HCl the rate was found to be first-order from 1 to 10 days. Slight deviations were found in the first day, indicating that some constituent of the sample (about 0·4 %) hydrolysed more rapidly; and the apparent rate became a little slower after 10 days. This was shown to be due to the formation of chloroform-soluble products by secondary reactions from the primary products of hydrolysis. However, the first

term of the equation was a very good approximation to the truth in very dilute solution, and, applied to the results, gave $k_1 = 4{\cdot}50 \pm 0{\cdot}03 \times 10^{-5}$ min.$^{-1}$.

The experiment was repeated at a higher concentration (0·47 %). The rate was initially 28 % greater; it decreased to that of the reaction in more dilute solution as the reaction proceeded, and k_2A^2 became less. Thus the equation fitted the observed results in the form

$$-\mathrm{d}A/\mathrm{d}t = 4{\cdot}50\ A(1 + 0{\cdot}2824\ A/A_0) \times 10^{-5}. \quad (3)$$

Now the rate of formation of the stable inhibitor is given by:

$$\mathrm{d}I/\mathrm{d}t = k_3A^2 - k_4I, \quad (4)$$

where k_3 is the constant for the rate of formation of the stable inhibitor (and is less than k_2, which is the constant for the formation of both inhibitors) and k_4 is the constant for hydrolysis of the inhibitor. In pure solution $k_4 = 3{\cdot}66 \times 10^{-5}$ min.$^{-1}$ (Table 8). By a similar experiment it was shown that when 64·4 % of a 0·47 % solution of thiolate had decomposed, $k_4 = 7{\cdot}26 \times 10^{-5}$ min.$^{-1}$. Thus k_4 varied with A. It was assumed that the relationship was linear.

Thus from (3) A could be found by computation at various times. Hence, by computation, and introducing the values for k_4, I could be found from (4). Now k_3 could not be determined independently, but the only effect of arbitrary changes in k_3 is to change the units in which I is calculated, so that k_3 was taken arbitrarily to be unity. Then $I_{\mathrm{calc.}}$ was a constant factor times the true value of I. This factor was chosen to give the best fit in the following experiment.

The concentrations of both inhibitors in a solution of thiolate in 0·005 N-HCl (0·47 %) were found at various times, and the concentration of the stable inhibitor is compared with those calculated in Table 10.

The agreement between the experimental and calculated concentrations was satisfactory except that the experimental values were somewhat high at the beginning. This indicates that the reactions postulated were the only major reactions leading to the stable inhibitor, but that some other minor reactions might have contributed.

The stable inhibitor accounts for nearly the whole difference between the rates at the two thiolate concentrations. From the residual difference the I_{50} of the unstable inhibitor is found to be $< 10^{-9}$ M. The higher concentration is near saturation, which may affect the rate. Even so, the I_{50} is unlikely to be greater than 5×10^{-9} M.

OO-*Dimethyl* S-*ethylsulphinylethyl phosphorothiolate*

On storing the purified compound for several months at room temperature its toxicity increased, the LD_{50} by intravenous injection decreasing from 48 to 5 mg./kg. Greater increases were found after heating. The increases were due entirely to compounds not extractable from water by chloroform.

Table 10. *Concentrations of* OO-*dimethyl* S-*ethylsulphoniomethylethyl phosphorothiolate produced by the action of* 0·005 N-HCl *on the sulphide at* 4·70 *mg./ml. concentration at* 37°

A nearly saturated solution of *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate was left for one day at about 20° in 0·005 N-HCl to remove any unstable impurities. The compound was purified by extraction into benzene, and a solution (0·47 %) in 0·005 N-HCl prepared rapidly from it. The solution was held at 36·98 ± 0·03° throughout the experiment. At set times samples were removed and extracted rapidly 3 times with $CHCl_3$, and the I_{50} dilutions of the aqueous layers determined immediately. The aqueous layers were then stored and the I_{50} dilutions redetermined 1 and 2 days after sampling. From the concentrations found at 1 and 2 days the concentration of the stable inhibitor (sulphoniothiolate) at the times of sampling were calculated (penultimate column). The concentrations of the unstable inhibitor were calculated from the difference between the observed I_{50} dilutions at sampling and those calculated for the stable inhibitor. In the last column are given the theoretical concentrations of the stable inhibitor, computed as described in the text. Some I_{50} dilutions were obtained from I_{50} graphs showing marked scatter. These are less accurate, and are marked ~. Other results are believed to be reliable to better than ±10 %. The concentrations of transient inhibitor after 3094 min. were less than the experimental errors of the determinations, so no values are given.

	Concn. of inhibitor (arbitrary units)					
	Observed					
Time (min.)	Total at sampling	1 day later	2 days later	Transient inhibitor	Sulphoniothiolate at sampling	Sulphoniothiolate (theor.)
0	175	7	—	170	7	0
237	5 400	870	850	4430	918, 974	830
1 414	8 750	4 800	3 840	3800	4 950, ~4 300	4 520
3 094	11 500	8 200	8 280	2100	~8 700, 9 420	8 670
8 654	15 100	13 600	11 750	—	14 700, 13 800	15 400
11 550	17 000	14 300	13 260	—	~15 600, 15 800	16 000
18 720	—	13 770	11 290	—	13 800, 14 100	13 800
21 530	13 400	11 040	—	—	12 100	12 300
31 690	7 370	—	—	—	~7 000	7 070

No detectable toxic derivatives were found on storing an aqueous solution for one day.

The sulphoxide cannot form a methylsulphonium derivative directly but, if reduction to the thiolate takes place, the thiolate can be methylated in the usual way. The oxygen acceptor could be either the sulphoxide itself:

$$2(MeO)_2PO\cdot S\cdot CH_2\cdot CH_2\cdot SO\cdot Et \rightarrow (MeO)_2PO\cdot S\cdot CH_2\cdot CH_2\cdot SO_2\cdot Et + (MeO)_2PO\cdot S\cdot CH_2\cdot CH_2\cdot SEt \quad (5)$$

or degradation products.

We therefore looked for thiolate in aged and heated samples, using successive partitioning. The procedure was exactly as described under Table 3, except that the light petroleum layers were subjected to successive partitioning with water. Both from the aged sample and from one heated for 16 hr. at 76° about 5% of the total phosphorus was extractable by light petroleum, with a light petroleum–water partition coefficient of $1{\cdot}60 \pm 0{\cdot}05$, in excellent agreement with that of the thiolate.

The proportion of sulphone was unchanged. This could imply either that sulphone was not formed from sulphoxide by the above reaction or that it was formed but was unstable, so that the final concentration of sulphone was similar to the initial concentration. It was shown by adding sulphone to the sulphoxide, heating for 16 hr. at 76° and analysing the product by successive partitioning, that the sulphone was not decomposed appreciably. Thus the oxygen acceptor was not the sulphoxide sulphur. In all experiments more phosphorus was found as compounds inextractable by chloroform than was found as thiolate; so that there were no inconsistencies in the hypothesis that degradation products were the oxygen acceptors.

OO-Dimethyl S-ethylsulphonylethyl phosphorothiolate

Heating the purified compound for 16 hr. at 76° left it unchanged, except for the production of 1% of compounds inextractable by chloroform. The aqueous fraction after chloroform extraction was not toxic.

Toxic compounds were formed from the original sample on heating. They were therefore produced from impurities, probably the sulphoxide. The purified compound did not form toxic derivatives.

Toxic properties of the compounds

The I_{50} values, and the oral and intravenous toxicities to rats of the purified compounds, are given in Table 11, those precautions being taken in their determination that are indicated by the results of previous sections.

OO-Dimethyl *S*-ethylthioethyl phosphorothiolate and its thionate isomer reacted too rapidly with water to be applied as aqueous solutions or suspensions, and were therefore injected, undiluted, by micro-syringe. The remaining compounds were made up as aqueous solutions immediately before use. A possible impurity, in addition to those

Table 11. *Toxic properties of the compounds*

95% fiducial limits are given below values obtained on sixteen rats. In those instances marked (–) all four rats died at twice the dose at which all four survived, so that no limits can be calculated.

Compound	LD_{50} values to white rats (mg./kg.) Intravenous	Oral	I_{50} (M)
OO-Dimethyl *S*-ethylthioethyl phosphorothiolate	64·6, ♀ (54·4–76·8)	62·5, ♀ (45·5–85·9)	$6{\cdot}5 \times 10^{-5}$
OO-Dimethyl *O*-ethylthioethyl phosphorothionate	216, ♀ (–)	676, ♀ (–)	—
OO-Dimethyl *S*-ethylsulphinylethyl phosphorothiolate	47·2, ♂ (–)	65, ♀ (54·4–76·8)	$4{\cdot}1 \times 10^{-5}$
OO-Dimethyl *S*-ethylsulphonylethyl phosphorothiolate	21·7, ♀ (17·5–27·0) 23·7, ♂ (–)	32·4, ♀ (27·3–38·5)	$2{\cdot}3 \times 10^{-5}$
OO-Diethyl *S*-ethylthioethyl phosphorothiolate	—	—	$5{\cdot}4 \times 10^{-6}$
OO-Dimethyl *S*-ethylsulphonioethylmethyl phosphorothiolate	0·0615, ♀ (0·0523–0·0724)	9·8,* ♀	$3{\cdot}9 \times 10^{-3}$
OO-Diethyl *S*-ethylsulphonioethylmethyl phosphorothiolate	0·0160, ♀ (0·0143–0·0180)	20,† ♀	$4{\cdot}7 \times 10^{-3}$
OO-Diethyl *S*-ethylsulphoniodiethyl phosphorothiolate	0·010,* ♀	—	$2{\cdot}6 \times 10^{-3}$

* Approximate values obtained on 6–8 rats.

† This dose killed in two days, higher doses considerably more rapidly. Doses of 5 mg./kg. showed no effect.

already discussed, in all the dimethyl compounds before purification was tetramethyl pyrophosphate. Except for the thionate, every compound was left long enough in water during purification to degrade this practically completely. The very low toxicity of the thionate and the peculiar symptomatology it produced, described below, showed that tetramethyl pyrophosphate was absent from this compound also.

The I_{50} of the thionate could not be determined, because it reacted so rapidly with water. That of the thiolate isomer was determined by making up an aqueous solution of about 2×10^{-4} M very rapidly in water, and using it immediately. At this dilution the formation of methylsulphonium derivatives is too slow to influence the results appreciably.

Intravenous injection of all compounds except the thionate produced typical symptoms of anticholinesterase poisoning in rats at doses close to the LD_{50}. The thionate, however, produced immediate inco-ordination, followed by accelerated respiration and weakness lasting several hours. Lethal amounts produced deep anaesthesia (with occasional jerking) which lasted about 30 min. Respiration then became more and more rapid until the animal died within 1–2 hr. At no stage were typical anticholinesterase symptoms observed. A similar, but weaker, anaesthetic stage was observed in rats injected intravenously with the thiolate isomer, but this was followed by the typical symptoms of anticholinesterase poisoning, from which the animals died. In neither case did injection of about 40 times the organic solvent in the samples induce any noticeable symptoms. Oral administration produced typical anticholinesterase symptoms with all compounds including the thionate. The duration of symptoms was markedly longer than after intravenous injection. The methylsulphonium compounds of both dimethyl and diethyl phosphorothiolate were very much less toxic orally than intravenously, although no accurate figure was obtained for the diethyl homologue. The symptoms from this compound developed exceptionally slowly, leading with fatal doses only to death after 24 hr. Bracketing the LD_{50} was therefore very difficult, especially as we had only a few milligrams of the compound.

A detailed study of the toxic action of some of these compounds is given by Vandekar & Heath (1957).

DISCUSSION

Although the tests of purity in the first section of the results were considerably more sensitive than the combination of elementary analysis and infrared spectroscopy used by most of those working with organophosphorus compounds, they were necessarily incomplete. The impurities which may not have been revealed were the *S*-methyl isomers. It is possible that the rates of recovery of cholinesterase inhibited by the various compounds would have indicated the presence of these compounds, as the rate of recovery is dependent upon the less easily replaced groups, i.e. R and R′ in RR′PO·X. The sensitivity of this test would depend on the relative I_{50} values of the isomers, about which nothing is known. However, the study of the reactions undergone by the compounds constituted a further series of tests of purity, as we were not forced to postulate impurities in order to account for the numerous products revealed. We therefore feel reasonably confident in treating the compounds as pure in the discussion that follows.

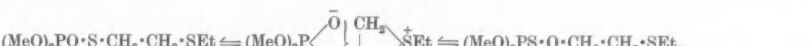
The reactions undergone by samples of four of the compounds in water and on storage profoundly affected their toxicological and biochemical properties, and presented some interesting chemical features.

In dilute solution in water *OO*-dimethyl *O*-ethylthioethyl phosphorothionate reacted very rapidly (half-life, about 10 min. at 37°). The main products were ions, with no detectable toxic properties, and the thiolate isomer in 30% yield. A very powerful, positively charged anticholinesterase was also formed, which was unstable in water (half-life, 100 min. at 37°). Much more of this was formed by adding thiolate to the thionate (Table 9); so, whatever its structure, it appears certain that it was produced by a reaction between thionate and thiolate.

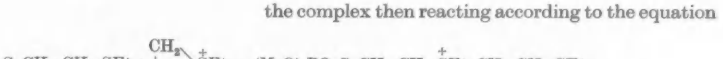
Both *OO*-dimethyl and *OO*-diethyl *S*-ethylthioethyl phosphorothiolate underwent reactions in water and on storage. The main products were non-toxic ions, but, in addition, self-alkylation led to alkylsulphonium derivatives. Various types of evidence established that the main toxic product from the dimethyl homologue was its methylsulphonium derivative, and that in water it was produced by second-order self-alkylation [see equation (1) above]. This compound was very toxic, administered by intravenous injection (LD_{50}, 0·062 mg./kg.), a powerful inhibitor of cholinesterase (Table 11) and fairly stable in water (Table 8). The diethyl homologue underwent similar reactions at about one-tenth the rate. Simultaneously, however, the dimethyl compound produced an unstable inhibitor very similar to that produced by mixtures of thionate and thiolate. This (see Table 10) contributed considerably more to the increase in the anticholinesterase activity of solutions of the dimethyl compound in the first few hours than the stable inhibitor formed simultaneously, but contributed relatively little after a few days storage. These results can be explained if the thiolate was in dynamic equilibrium with the thionate, with which it reacted to form the unstable inhibitor. The equilibrium would be heavily on the side of the thiolate.

Because of the complexity of these reactions it was essential to show that the reactions postulated could account quantitatively for the formation of the stable inhibitor. Only in this way could we feel any confidence that no reactions of any importance producing toxic derivatives had been obscured. Table 10 shows that the experimental results were in good agreement with theory, but indicates that some minor reaction may also have contributed during the first day or two.

The thiolates and thionate underwent similar reactions on storage. The main products were non-toxic ions and, from the thionate, its thiolate isomer. Against the structure proposed for the compound were the facts that it decomposed more rapidly in water than its thiolate isomer ($k = 7 \times 10^{-3}$ min.$^{-1}$ at 37° against $3{\cdot}7 \times 10^{-5}$ min.$^{-1}$ at 37°) and that it was a more powerful anticholinesterase ($I_{50} = 5 \times 10^{-9}$ M against $3{\cdot}8 \times 10^{-8}$ M). Another possibility which may avoid these difficulties is that the unstable inhibitor was formed by the reaction of the thiolate with the transition complex in the thiolate–thionate equilibrium. Thus, adapting the mechanism of isomerization suggested by Fukuto & Metcalf (1954) to equilibrium between thionate and thiolate, their equation becomes



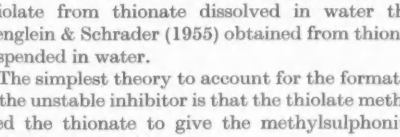
the complex then reacting according to the equation



Some alkylsulphonium derivatives were, however, formed, and in sufficient yield to increase the intravenous toxicities of old samples of the dimethyl thiolate and thionate 100-fold. *OO*-Dimethyl *S*-ethylsulphinylethyl phosphorothiolate on storage at room temperature and on heating was partially converted into the thioethyl compound, which then reacted to form alkylsulphonium compounds. The intravenous toxicity was thereby increased about 40-fold. The main products were non-toxic ions. Storage in 1 % aqueous solution for one day did not, however, affect the toxicity.

These reactions are in some ways surprising. Thus both of the thiolates, the thionate and the sulphoxide produced ionic non-toxic compounds in the absence of water. Water accelerated the reactions in the case of all but the sulphoxide, but was plainly not necessary. Also the dimethyl thiolate reacted much more slowly with water than the dimethyl thionate did, which is directly contrary to theoretical expectation and to what is observed with similar isomers (Heath, 1956). Work is proceeding at present on these problems, which appear to be related to the fact that we obtained smaller yields of thiolate from thionate dissolved in water than Henglein & Schrader (1955) obtained from thionate suspended in water.

The simplest theory to account for the formation of the unstable inhibitor is that the thiolate methylated the thionate to give the methylsulphonium derivative of the thionate:



More detailed study was impossible because, owing to its instability, we could not separate enough to carry out analyses. There is therefore some doubt about its structure, and we shall refer to it below as the unstable inhibitor.

The reactions yielding toxic compounds are probably not confined to compounds of the types described in this paper. These reactions are self-alkylations, which implies that dialkyl phosphate esters can act as alkylating agents (Heath, 1957), though many may be very weak. Any dialkyl phosphate ester containing an acceptor atom, such as binary sulphur or ternary nitrogen, will therefore alkylate itself. It is probable that the acceptor atoms have to be in certain positions in the molecule relative to the phosphorus atom if the products are to be more toxic than the parent compounds, but, whether the products are more or less toxic than the parent compounds, the effect is a change in biochemical and toxicological properties on storage which may lead to inconsistent results. It is advisable, therefore, to check the purity frequently of any compound which might undergo such reactions during biochemical work with it.

We now consider the effects on biochemical and toxicological properties of the reactions already discussed, and some of their implications.

The intravenous toxicities of dimethyl thiolate and thionate increased rapidly on storage at room temperature. Although no exact experiments have been carried out, it appears likely that the original toxicity of the pure compound was at least doubled in a week. The conversion into alkylsulphonium derivatives far outweighed in its effect conversion into inert ionic products, although this was more rapid. For example, a 2-year-old sample of the

thiolate contained 56 % of inert ionic products, and 4 % of sulphoniothiolate and was about 100 times as toxic as the pure compound. In water the thionate increased in toxicity very rapidly. The increase was dependent on the concentration, and in a 0·2 % solution was over 100-fold. The increase in toxicity of thiolate solutions at 37° was slower, and was in the first day mainly to be accounted for by the formation of the unstable inhibitor. After this the stable inhibitor became the more important. In nearly saturated solutions (1 %) the intravenous toxicity was increased by a factor of a hundred. The toxicity of the diethyl thiolate increased relatively little on storage and in water, because the decomposition of the compound to inert compounds nearly kept pace with the formation of alkylsulphonium derivatives. The symptomatology was, however, changed somewhat.

In biochemical work with organophosphorus compounds the high toxicities of samples contaminated with minute traces of sulphonium derivatives underline the necessity for proving that no physical or physicochemical methods of fractionation can change the properties of samples under test. Aldridge & Davison (1952) had already shown that many results initially attributed to various diethyl phosphate esters in fact arose from contamination with tetraethyl pyrophosphate. Both in their work and ours no purely chemical or physical method of analysis existed that was sensitive enough to detect impurities in quantities able to control completely any biochemical observation made. Probably the group of compounds we have studied are extreme in their behaviour, but the need for caution is obvious in interpreting results obtained even with compounds subjected to stringent tests of purity.

The reactions of the dimethyl thiolate and thionate are expected to lead to difficulties in obtaining reproducible results with the insecticide Metasystox, which is a mixture of the isomers. The history of a sample of Metasystox from the factory to its degradation in the crop reveals a very large number of factors which are essentially uncontrollable and which may not influence the action of other systemic insecticides to the same extent. The time for which and the temperature at which the sample is stored will affect its constitution, as isomerization of thionate to thiolate, breakdown to non-toxic ionic products, and self-alkylation to toxic products take place on storage. Similar factors will influence the same reactions in the spray tank when the spray is made up. During and after spraying the aqueous spray is concentrated, leading to a considerable acceleration in the formation of toxic derivatives. How far these reactions proceed will depend on the rates at which the various constituents are absorbed by the leaves, or dissolved in or adsorbed on non-polar constituents on the leaf surface, factors in turn dependent upon such variables as humidity, temperature and wind-speed. What is found inside the leaf will depend on the relative rates of absorption of the compounds, and their relative stabilities in the plant in question. Possibly also the plant itself may methylate these compounds to sulphonium derivatives. We conclude that some variations in toxicity to insects may be observed under different conditions. Dr Harlow and Dr Hopf (I.C.I. Ltd., Jealott's Hill Research Station, Bracknell, Berks) have informed us privately that our sample of the sulphoniothiolate showed only weak contact action against some insect species. Fukuto, Metcalf, March & Maxon (1955*b*) found, however, that the methylsulphonium derivative of Isosystox exerted systemic action, and Heath *et al.* (1955) found, in crops treated with Isosystox, a non-chloroform-extractable anticholinesterase, which it is now reasonable to suppose was its ethylsulphonium derivative.

The presence of alkylsulphonium derivatives in crops at the time of harvesting may lead to an overestimate of the toxic content of the crop when analyses are carried out by determinations of the anticholinesterase activity of plant extracts. These derivatives are very powerful anticholinesterases, but relatively non-toxic when taken by mouth (Table 11). In this context the margin of safety is increased.

These considerations are probably relatively unimportant with the sulphoxide, which produced toxic derivatives relatively slowly on storage and in water, and probably do not apply to the sulphone, which appeared to be a stable compound.

On the assumption that our compounds were pure we can discuss their toxic properties as summarized in Table 11.

The intravenous toxicity increased up the dimethyl phosphate series thionate, thiolate, sulphoxide and sulphone, as up the diethyl phosphate series of homologues (Fukuto, Metcalf, March & Maxon, 1955*a*). The compounds were, however, markedly less toxic than their homologues, and weaker inhibitors of cholinesterase. The oral and intravenous LD_{50} values are very similar, in spite of the slower rate of absorption after oral administration. This suggests either that the compounds persisted in the body (Vandekar & Heath, 1957) or that they were converted into more toxic compounds.

The sulphonium derivatives of the thiolates were all highly toxic by intravenous injection, and were powerful inhibitors of cholinesterase. There is marked similarity between the $OP \cdot S \cdot CH_2 \cdot CH_2 \cdot \overset{+}{S}R_2$ grouping in these compounds and

$$OC \cdot O \cdot CH_2 \cdot CH_2 \cdot \overset{+}{N}Me_3$$

in acetylcholine, which probably results in strong adsorption on the anionic site of cholinesterase.

orally, they were relatively much less toxic, perhaps because their ionic charge interfered with absorption. It was impossible to determine the LD_{50} values of the unstable inhibitor, as not enough was separated. It must, however, have been of comparable toxicity to the other methylsulphonium compounds, as the increase in water in the toxicity of aged samples of thionate and thiolate was considerable (20–22 mg./kg.), although in similar experiments with pure compounds the phosphorus content of the compound was undetectable (Table 9). As already stated, the I_{50} of the unstable inhibitor was at most 5×10^{-9} M.

The oral LD_{50} values of stored thiolate samples containing a few per cent of the sulphonium derivatives were similar to that of the pure thiolate. The effect of the impurities was obvious only when samples were injected intravenously or tested against cholinesterase *in vitro*.

The thionate did not produce cholinergic symptoms when given intravenously, but produced an anaesthetic affect. This was also observed with the thiolate, though in this instance cholinergic symptoms developed later. The observations led to the work described by Vandekar (1957), who showed that several phosphate esters of low toxicity produce anaesthetic effects. Given orally, both compounds produced cholinergic symptoms only. The metabolic reactions that these compounds undergo *in vivo* are being investigated.

It should be plain from the preceding discussion that the characteristic and marked differences in the properties of the compounds, both chemical and biochemical, appeared only because of the care taken in purifying them.

SUMMARY

1. *OO*-Dimethyl *S*-ethylthioethyl phosphorothiolate and its diethyl homologue, thionate isomer, sulphoxide and sulphone have been purified by solvent-extraction procedures, and their purities tested. Their oral and intravenous LD_{50} values to rats and I_{50} values against cholinesterase have been determined.

2. On storage or in water the intravenous toxicities of all but the sulphone increased 40- to 100-fold, owing to the formation of alkylsulphonium derivatives. Thus the thioethyl thiolates alkylated themselves to alkylsulphonium thiolates; the dimethyl thioethyl thiolate appeared to be in dynamic equilibrium with its thionate isomer, with which it reacted to give a sulphonium derivative; and the sulphoxide gave some thioethyl thiolate, which was also alkylated to sulphonium compounds. The sulphone was stable.

3. The alkylsulphonium compounds were very powerful anticholinesterases, very toxic by intravenous injection (LD_{50} values of 0·01–0·06 mg./kg.), but much less toxic orally.

4. Sulphonium compounds present in amounts below the limits of physical and chemical detection can interfere with biochemical and toxicological observations. The importance of using biochemical tests of purity is stressed.

5. The effects of these facile reactions on the study of biochemical and toxicological effects are pointed out with reference to insecticidal action, analysis of residues in crops and the practical precautions which have to be taken to obtain reproducible LD_{50} values by intravenous injection.

We thank Dr G. S. Hartley, Fisons Pest Control Ltd., Little Chesterford, Essex, and Dr G. Schrader, I.G. Farbenindustrie, Leverkusen, for compounds; Dr I. K. H. Otter, Fisons Pest Control Ltd., Little Chesterford, for the chromatographic methods; Miss J. Lewis, National Institute of Medical Research, Mill Hill, for carrying out elementary analyses; Dr P. A. Harlow and Dr H. S. Hopf, of Imperial Chemical Industries Ltd., Bracknell, Berks, for some entomological testing; Dr A. H. Ford-Moore, Ministry of Supply, Porton, Wilts, and Mr J. F. Newman, I.C.I., Bracknell, for discussions on the properties of 'onium compounds; Dr J. M. Barnes and Dr W. N. Aldridge of this Unit for their interest in our work, and Mr C. R. Kennedy and Mr D. E. A. Groom for technical assistance.

REFERENCES

Aldridge, W. N. (1950). *Biochem. J.* **46**, 451.
Aldridge, W. N. (1953). *Biochem. J.* **54**, 442.
Aldridge, W. N., Berry, W. K. & Davies, D. R. (1949). *Nature, Lond.*, **164**, 925.
Aldridge, W. N. & Davison, A. N. (1952). *Biochem. J.* **51**, 62.
Collander, R. (1949). *Acta chem. scand.* **3**, 717.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Flynn, F. V. & Mayo, P. de (1951). *Lancet*, **2**, 235.
Fukuto, T. R. & Metcalf, R. L. (1954). *J. Amer. chem. Soc.* **76**, 5103.
Fukuto, T. R., Metcalf, R. L., March, R. B. & Maxon, M. G. (1955*a*). *J. econ. Ent.* **48**, 347.
Fukuto, T. R., Metcalf, R. L., March, R. B. & Maxon, M. G. (1955*b*). *J. Amer. chem. Soc.* **77**, 3670.
Gardner, K. & Heath, D. F. (1953). *Analyt. Chem.* **25**, 1849.
Heath, D. F. (1956). *J. chem. Soc.* pp. 3796, 3804.
Heath, D. F. (1957). *Nature, Lond.*, **179**, 377.
Heath, D. F. & Lane, D. W. J. (1952). *Brit. Pat.* 33119/52.
Heath, D. F., Lane, D. W. J. & Llewellyn, M. (1952). *J. Sci. Fd Agric.* **3**, 69.
Heath, D. F., Park, P. O. & Lane, D. W. J. (1955). *Phil. Trans.* **239**, 191.
Henglein, von A. & Schrader, G. (1955). *Z. Naturf.* **10**, 1.
Macpherson, J. B. & Johnson, G. A. (1956). *J. agric. Food Chem.* **4**, 42.
Otter, I. K. H. (1955). *Nature, Lond.*, **176**, 1078.
Seidell, A. (1941). *Solubilities of Organic Compounds*, 3rd ed. New York: D. van Nostrand.
Thompson, W. R. & Weil, C. S. (1952). *Biometrics*, **8**, 51.
Vandekar, M. (1957). *Nature, Lond.*, **179**, 154.
Vandekar, M. & Heath, D. F. (1957). *Biochem. J.* **67**, 202.
Weil, C. S. (1952). *Biometrics*, **8**, 249.

The Reactivation of Cholinesterase after Inhibition *in vivo* by some Dimethyl Phosphate Esters

By M. VANDEKAR* and D. F. HEATH
Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey

(*Received* 18 *December* 1956)

It is generally believed that inhibition of cholinesterase by organophosphorus compounds is the result of phosphorylation of the enzyme's active centre. Aldridge (1953*a*) showed that reactivation of the phosphorylated enzyme by hydrolysis is more rapid when cholinesterase is inhibited by certain dimethyl phosphate esters *in vitro* than when it is inhibited by the corresponding diethyl esters. Davison (1955), studying the return of cholinesterase activity *in vivo* after inhibition with different organophosphorus compounds, found that reactivation of inhibited rat-brain cholinesterase took place at the same rate as *in vitro*. A change of slope at about 55% recovery was observed, suggesting that the remainder of the inactivated enzyme is more permanently inhibited.

Some preliminary observations showed that *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate and some of its derivatives, injected into animals, produced prolonged symptoms with delayed recovery. This was inexplicable if the inhibited enzyme recovered its activity at the high rate indicated by Aldridge's (1953*a*) experiments *in vitro*, and we were therefore led to investigate the action of these compounds in more detail. We have shown that they persist in the body and that, in consequence, cholinesterase is inhibited in a form from which it cannot be reactivated at an appreciable rate. A brief communication has already been made outlining the results (Vandekar, 1957). The work carried out *in vitro* by Wilson (1955), Davies & Green (1956) and Hobbiger (1956) suggests how this comes about. They showed that the degree of reactivation of inhibited enzyme by nucleophilic reagents is inversely related to the time of contact between enzyme and inhibitor. It has been suggested that this inhibition is a two-stage process, an initial form of inhibited enzyme being converted at a first-order reaction rate into a second form which can no longer be reactivated. This conversion occurs more rapidly with dimethyl- and di*iso*propyl- than with diethyl-phosphorylated enzyme.

In the present work we have shown that *in vivo*, also, the two-stage process of inhibition occurs after administration of some dimethyl phosphate esters. The rapidly-reversible first stage of inhibition changes into the non-reversible stage whenever prolonged contact between inhibitor and enzyme takes place. Thus non-reversible inhibition may be produced either by a rapid injection of an inhibitor which persists in the body or by artificially prolonged administration of a non-persistent inhibitor such as dimethyl *p*-nitrophenyl phosphate.

We have used for this study *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate and some related compounds, and compared their toxic action with that of dimethyl *p*-nitrophenyl phosphate (Me-E600) and a diethyl phosphate inhibitor. The reproducibility of the toxic properties of these compounds and of the differences between them result from the care taken in their purification, as described by Heath & Vandekar (1957).

MATERIALS AND METHODS

The compounds used for this study are named in Table 1.

Dimethyl *p*-nitrophenyl phosphate (Me-E600) was prepared as follows: dimethyl phosphorochloridate (42 g.), *p*-nitrophenol (41·8 g.), Na_2CO_3 (31·8 g., dried for 0·5 hr. at 160°), acetone (200 ml.) and ethyl methyl ketone (100 ml.) (both dried over anhyd. Na_2SO_4) were refluxed gently with brisk stirring for 3 hr. The product was cooled, filtered, and concentrated *in vacuo*. The residue was shaken with Na_2CO_3 solution (15 g./500 ml. of water) and extracted with benzene. The benzene extract was shaken in turn with Na_2CO_3 solution to remove any remaining *p*-nitrophenol and twice with water, and immediately concentrated *in vacuo*. The product was purified by fractionation, b.p. 150° at 0·20 mm. Hg. The compound contained 0·07% of free *p*-nitrophenol and liberated *p*-nitrophenol equivalent to 96·5% of the theoretical on hydrolysis. The only likely impurity other than solvent is tetramethyl pyrophosphate. To remove any present, aqueous solutions used were stored for 2 hr. at room temp. before use.

The other compounds were purified as described by Heath & Vandekar (1957).

All compounds were stored in the dark at −30° between experiments. To avoid conversion in water into other toxic compounds *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate was injected without dilution; other compounds (except Me-E600) were diluted with water immediately before use. Except when stated otherwise, the doses injected were those given in the second column of Table 1.

Estimation of the recovery of brain and erythrocyte cholinesterase in vivo. Male and female albino rats (180–250 g.)

* Present address: Institute for Medical Research, Zagreb, Yugoslavia.

were given organophosphorus compounds via the tail vein. The doses were close to the LD_{50} and invariably produced severe symptoms. At selected times animals were killed by coal gas. On death, blood (5–8 ml.) was collected from the vena cava into 0·2 ml. of 1% heparin, and erythrocytes were washed twice with cold buffer (0·0357 M-$NaHCO_3$, 0·164 M-NaCl and 0·040 M-$MgCl_2$, freshly prepared from stock solutions, and cooled to 5° before use).

Estimations of cholinesterase activity were carried out on 1 ml. of brain suspension or on washed erythrocytes corresponding to 1·5 ml. of blood in a Warburg apparatus at 37° with an atmosphere of N_2+CO_2 (95:5). Acetylcholine chloride was used as substrate at a final concentration of 0·0138 M. Gassing and equilibration time in the bath did not exceed 15 min.

Estimation of persistence of anticholinesterase activity of serum in rats. This was done at selected intervals after a single intravenous injection, when animals were killed by coal gas, and blood was collected as described above. Serum was separated and 2·0 ml. added to sedimented washed normal rat erythrocytes and incubated for 1 hr. at 37°. The erythrocytes were then washed twice with buffer, and the cholinesterase activity was estimated as above. The results are expressed as percentages of the activity of erythrocytes treated in the same way with serum obtained from normal rats.

Slow intravenous infusion of Me-E 600. One day before infusion, fine polythene canulae were inserted into the vena jugularis of male rats (225–275 g.) anaesthesized with ether. Different concentrations of Me-E 600 in saline were infused at 1·0 ml./hr. by means of a constant-injection apparatus as described by Church & Ridge (1955). During infusion, animals were kept in small cages, which allowed undisturbed feeding and drinking but prevented the animals from turning round. The symptoms were followed during each infusion and, at the onset of death, the positions of canulae were checked by injection of dye or air through them. Results were considered valid when no leakage of the dye (or air) was observed in the area surrounding the place of insertion of the canulae. In a satisfactory experiment the dye (or air) went only through the heart's auriole and out through the lower vena cava, which was cut. Such precautions were necessary because the convulsions which might take place during infusion sometimes displaced the canula.

The brain was dissected out and suspended in cold buffer in order to determine the return of cholinesterase activity *in vitro*.

Return of cholinesterase activity in vitro *in the brains of rats treated with Me-E 600.* The rate of return of cholinesterase activity was determined on 1 ml. of brain suspension by the method of Aldridge (1953*a*), in which excess of inhibitor is removed by a preparation of A-esterase. The activity obtained after 4 hr. incubation at 37° was taken as 89·4% of complete reactivation, a value calculated from the half-life for the reaction of 1·23 hr., obtained by experiments where inhibition was produced *in vitro* by incubation for 15 min. only at 37°. In this time very little of the enzyme is inhibited irreversibly.

RESULTS

Dimethyl phosphate esters

Response to a single acute dose. After intravenous administration of doses close to the LD_{50} all compounds produced typical symptoms of acetylcholine poisoning. Marked differences, however, were observed in the onset and duration of symptoms. The dimethylsulphonium compound and Me-E 600 produced rapid onset of symptoms and, if rats survived the critical first few minutes, they began to recover very soon. Within 1 hr. of sublethal intravenous injection they showed no symptoms except perhaps an occasional fasciculation.

On the other hand, the sulphide and its two oxidative products, the sulphoxide and sulphone, produced symptoms which developed relatively slowly. Pronounced fasciculations, tremor, gasping, salivation and chromodacryorrhea lasted for 3–4 hr., and moderate fasciculations and prostration were present for more than 5 hr. (Table 1). Even 24 hr. later, the animals were still weak and dirty, with staring coats, and had lost 10–15% of their weight.

Table 1. *Duration of symptoms after sublethal intravenous injection of different dimethyl phosphate esters and one diethyl phosphate ester into rats*

Except for Me-E600, the values for intravenous LD_{50} are taken from Heath & Vandekar (1957).

	Intravenous LD_{50} (mg./kg.)	Amount administered intravenously (mg./kg.)	Duration of symptoms (hr.)
$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SEt$ (Sulphide) (*OO*-dimethyl *S*-ethylthioethyl phosphorothiolate)	64·6 (♀)	50·0 (♀)	> 5
$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SO{\cdot}Et$ (Sulphoxide) (*OO*-dimethyl *S*-ethylsulphinylethyl phosphorothiolate)	47·2 (♂)	40·0 (♂)	> 5
$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}SO_2{\cdot}Et$ (Sulphone) (*OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate)	23·7 (♂) 21·7 (♀)	— 17·3 (♀)	— > 5
$(MeO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{S}MeEt$ (Dimethylsulphonium) (*OO*-dimethyl *S*-ethylsulphonioethylmethyl phosphorothiolate)	0·0615 (♀)	0·040 (♀)	Approx. 1
$(MeO)_2PO{\cdot}O\text{-}p\text{-}C_6H_4{\cdot}NO_2$ (Me-E 600) (dimethyl *p*-nitrophenyl phosphate)	0·410 (♀)	0·30 (♀)	1 or less
$(EtO)_2PO{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}\overset{+}{S}MeEt$ (Diethylsulphonium) (*OO*-diethyl *S*-ethylsulphonioethylmethyl phosphorothiolate)	0·01 (♀)	0·01 (♀)	4–5

Reactivation of cholinesterase in vivo. The difference in the duration of symptoms between the two groups of compounds suggested that the inhibited enzyme was reactivated at different rates. We therefore investigated the rate at which cholinesterase activity returned *in vivo* in the brain and erythrocytes of rats given single injection of these two groups of compounds.

The activity of brain and erythrocyte cholinesterase was estimated at selected intervals after injection of sublethal doses. As shown in Fig. 1*a* intravenous injection of the dimethylsulphonium derivative produced a considerable inhibition of erythrocyte cholinesterase 10 min. after injection, after which enzyme activity returned rapidly. Brain-cholinesterase activity was only slightly reduced. Similarly, Fig. 1*b* shows that erythrocyte cholinesterase inhibited by Me-E 600 recovers its activity at the same rapid rate. This compound also inhibited brain cholinesterase, the activity of which returned just as rapidly.

Sulphide, sulphoxide and sulphone reduced both brain- and erythrocyte-cholinesterase activity to 30 % of normal, but in this group the reduction was maintained for the first 24 hr. After this period the activity returned slowly and reached about 75 % in 28 days (Fig. 1*d–f*).

Reactivation of cholinesterase in vitro. These results showed that the different durations of symptoms are paralleled by different rates of reactivation of the enzyme *in vivo*. The high rates of reactivation found after injection of Me-E 600 and the dimethylsulphonium compound are consistent with the rates of reactivation *in vitro* found by Aldridge (1953*a*) for dimethylphosphorylated enzymes. The sulphide, sulphoxide and sulphone inhibit far more permanently, which might imply that not all dimethyl phosphate esters are able to produce reversible inhibition. We therefore determined the rate of reactivation of cholinesterase inhibited *in vitro* with all five compounds. Aldridge's technique was used, in which the inhibitor and rabbit erythrocytes are left in contact for 15 min. only at 37°, after which the return of cholinesterase activity is followed. There was no distinction between the five compounds when tested in this way. The return of activity took place at the same high rate (half-life, 1·3 hr.) characteristic of cholinesterase inhibited with dimethyl phosphate esters.

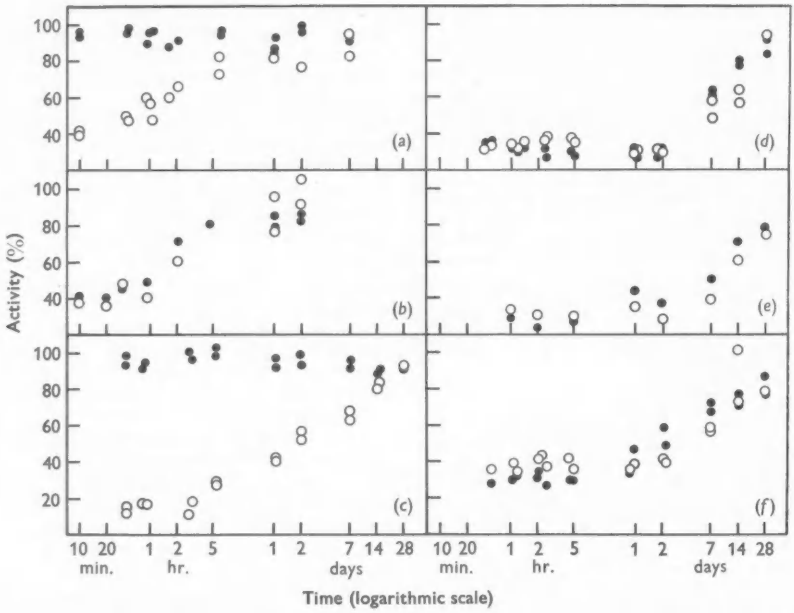


Fig. 1. Return of brain (●) and erythrocyte (○) cholinesterase activity in rats after single sublethal intravenous injection of (*a*) dimethylsulphonium derivative, (*b*) Me-E 600, (*c*) diethylsulphonium derivative, (*d*) sulphide, (*e*) sulphoxide and (*f*) sulphone. Each point represents one female rat except (*e*), where each point represents the mean of four male rats.

Persistence of inhibitor in the body. It seemed possible that the prolonged symptoms as well as the exceedingly slow rate of return of cholinesterase activity in animals following administration of the sulphide, sulphoxide and sulphone might be explained if these inhibitors persisted in the body. As no microchemical methods exist for determining these compounds and their products in animals, an attempt was made to detect the presence of free inhibitor by measuring the inhibitory activity of the circulating blood.

The anticholinesterase activity of serum was estimated at selected times after intravenous injection of sublethal doses of all five compounds (Fig. 2). After injection with the dimethylsulphonium derivative the rats' serum had lost most of its anticholinesterase activity within 2 hr. No inhibitor was detectable in serum 10 min. after injection with Me-E 600 (Fig. 2*a*). On the other hand, injection with sulphide, sulphoxide and sulphone produced an inhibitor which persisted for several hours in the blood. Some inhibitor was detectable even after 16 or 24 hr. (Fig. 2*b*).

These experiments showed that those dimethyl compounds which produced long-lasting inhibition of enzyme are those that persisted in the body.

Effect of slow infusion of Me-E 600. To provide further evidence that the production of permanent inhibition was due solely to the persistence of some dimethyl phosphate esters in the body, we investigated the type of inhibition produced when prolonged contact was arranged between a transient inhibitor and cholinesterase *in vivo*.

Me-E 600 was infused at a constant slow rate into rat's vena jugularis, and the return of cholinesterase activity was determined *in vitro* as described under Methods. Table 2 shows the amount of irreversible inhibition produced by such treatments, which is compared with that produced by a single intravenous injection into rat's tail vein. The rate of reactivation after a single rapid injection (which killed the animal within 5 min.) approached closely that of a first-order reaction such that, during the 4 hr. incubation, most of the enzyme was recovered.

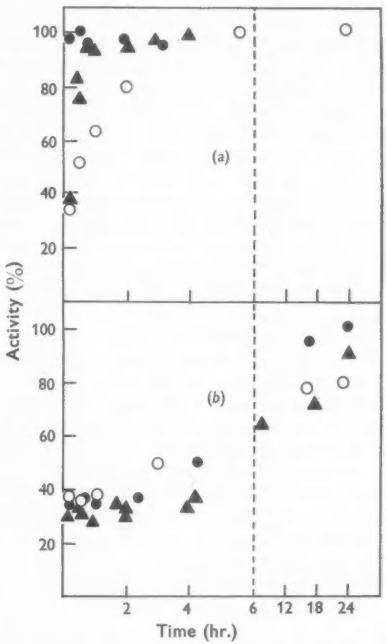


Fig. 2. Anticholinesterase activity of serum after intravenous injection of a single sublethal dose into male rats with (*a*) Me-E 600 (●), dimethylsulphonium derivative (○), diethylsulphonium derivative (▲), and (*b*) sulphide (○), sulphoxide (▲) and sulphone (●). Each point represents one rat.

Table 2. *Effect of slow intravenous infusion of Me-E 600 into rats*

Me-E 600 was infused at a constant slow rate into rat's vena jugularis or injected rapidly by a single intravenous injection into rat's tail vein. The return of cholinesterase activity was determined *in vitro* as described under Methods.

Rat no.	Rate and amount administered			Percentage of brain cholinesterase inhibited		
				At zero time	After 4 hr. of reactivation	Irreversibly
1	Rapid injection of 1·1 LD_{50}			63·6	10·2	3·9
	Infusion					
	Rate (LD_{50}/hr.)	Time of survival (hr.)	Dose tolerated (LD_{50})			
2	6·06	0·2	1·21	62·0	20·4	15·5
3	1·31	6·7	8·74	66·6	30·1	25·8
4	0·27	>34·0*	>7·43	82·3	66·8	65·0

* Killed by coal *gas*.

After slow infusion the amount of enzyme reactivated was much less, and the amount of irreversibly inhibited enzyme increased with the duration of infusion.

The total amounts of Me-E 600 tolerated at different rates of injection are shown in Fig. 3, and are expressed in terms of intravenous LD_{50} (= 0·410 mg./kg.). A decrease in the rate of infusion below 1·5 LD_{50}/hr. was accompanied by a remarkable increase in tolerance and survival time. All infused rats showed the typical symptoms of poisoning by acetylcholinesterase inhibitors, and passed through a stage in which they developed successively more severe symptoms, culminating in severe convulsions, dyspnoea with râles and tremor. Those that survived reached an 'equilibrium stage' which was characterized by constancy of symptoms (very pronounced tremor and fasciculations but only occasional slight convulsions) and lasted for several hours—only after which the animals became more and more prostrate, dyspnoea developing with increasingly laboured costal respiration. Death usually followed within half an hour after the onset of terminal dyspnoea, during which period fasciculations diminished and ceased. Convulsions (probably due to anoxia) and cessation of respiration always preceded death. The effects of different rates of infusion of other cholinesterase inhibitors have been studied also with results to be published in more detail elsewhere.

OO-*Diethyl* S-*ethylsulphonioethylmethyl phosphorothiolate*

Analogous experiments were carried out with the diethylsulphonium compound, to compare the previous results with those obtained on a diethyl phosphate ester.

After sublethal intravenous injection the onset of symptoms was rapid but their duration prolonged. The reactivation of cholinesterase inhibited *in vitro* took place at the slow rate typical of diethyl phosphorylated enzymes (Aldridge, 1953*a*). The rate of reactivation of erythrocyte cholinesterase *in vivo* was similar (Fig. 1*c*), showing that little or none of the enzyme had been inhibited irreversibly. Brain cholinesterase was not inhibited appreciably.

The compound did not persist in the serum (see Fig. 2*a*).

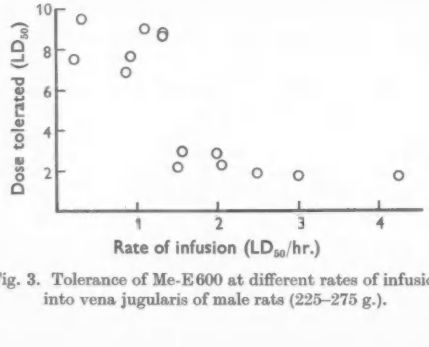


Fig. 3. Tolerance of Me-E 600 at different rates of infusion into vena jugularis of male rats (225–275 g.).

DISCUSSION

The dimethyl phosphate esters we have studied fall into two groups with very different biochemical actions *in vivo*: Me-E 600 and the dimethylsulphonium compound; and the sulphide, sulphoxide and sulphone.

Me-E 600 and the dimethylsulphonium derivative, when given intravenously in single sublethal injections, produced symptoms of short duration, which were shown to be associated with rapid recovery of cholinesterase activity *in vivo*. The inhibitor persisted in the blood only a very short time. These findings agree with those of Aldridge (1953*a*), who showed that cholinesterase inhibited *in vitro* by short contact with dimethyl phosphate esters recovered its activity rapidly when the inhibitor was removed.

The sulphide, sulphoxide and sulphone, on the other hand, administered in the same way, produced symptoms of long duration, which were associated with a very slow rate of return of cholinesterase activity *in vivo*. Inhibitors could be found in the blood in appreciable concentrations for several hours after injection. Delayed return of cholinesterase activity might be expected from the results obtained *in vitro* by other workers. Thus Wilson (1955) showed that prolonged contact between cholinesterase and dimethyl phosphate esters led to irreversible inhibition. Inhibition by organophosphorus esters is a two-stage process, in which the first, reversible, form of the inhibited enzyme is converted at a first-order reaction rate into a second form, which cannot be reactivated by nucleophilic reagents (Davies & Green, 1956; Hobbiger, 1956). Davies & Green (1956) have suggested that this reaction may be due to an initial phosphorylation of histidine followed by migration of the phosphoryl group to the hydroxyl group of serine. In our experiments prolonged contact between enzyme and inhibitor was achieved *in vivo*, apparently with the same results. The striking differences in the duration of symptoms observed in animals poisoned by single doses of dimethyl phosphate esters can therefore be reconciled with *in vitro* observations on the reactions of these compounds with cholinesterase. The difference between the two groups of compounds is determined by their different degrees of persistence in blood. If a non-persistent inhibitor is made to persist artifically by prolonged slow infusion, an irreversible inhibition of cholinesterase can be produced (see Table 2).

We cannot say whether those compounds producing long-lasting symptoms exert their action themselves, or whether they are converted *in vivo* into other active compounds. The sulphone is fairly stable in water and has no groups that we would expect to be readily attacked in the body. It is probable, however, that the sulphide and sulphoxide are oxidized *in vivo* to the sulphone, which is more toxic. March, Metcalf, Fukuto & Maxon (1955) have shown that such reactions take place *in vivo* with the diethyl homologues. Possibly the sulphide is converted into its much more toxic dimethylsulphonium derivative, a reaction known to take place rapidly *in vitro* (Heath & Vandekar, 1957). This reaction is bimolecular *in vitro*, and would be very slow at the concentrations present in the blood, unless other methylating agents act on the compound *in vivo*. We found that both the dimethyl- and diethyl-sulphonium derivatives did not inhibit brain cholinesterase when injected into animals (possibly because their ionic nature hindered passage through the blood–brain barrier). The sulphide inhibited brain cholinesterase considerably. Thus if the sulphide is converted into the sulphonium derivative this conversion must take place in the brain. The chemical conversions of these compounds *in vivo* are being studied at present.

When we consider why some compounds persist and not others, we can find a clear answer only in one instance. Me-E 600 is rapidly hydrolysed by A-esterase, which is present in serum (Aldridge, 1953*a*, *b*), and hence cannot be found in the blood 10 min. after injection. The sulphonium compounds do not persist *in vivo*, although they are fairly stable *in vitro*. They may be removed either by losing methyl groups to potential acceptors normally present in the body, with the formation of the much less toxic sulphides, or, in view of the minute quantities injected, they may be excreted rapidly in urine, removed by reaction with other esterases or irreversibly adsorbed on surfaces in the body. The sulphide and sulphoxide are probably converted into the sulphone. Apparently there is no biochemical system *in vivo* that is capable of degrading the sulphone rapidly. The relatively large amounts injected and conversions into still more toxic compounds (sulphone and possibly sulphonium compound) may also contribute to the persistence. Until much more is known of the factors involved, it will be impossible to predict the persistence of any given compound which is fairly stable in water; also there is no reason to believe that persistence *in vivo* is rare. Consequently, how long a compound persists must be found in every instance. Where it persists, the possibility of accumulation during repeated exposures, as well as the effect on the type of inhibition produced, must be borne in mind.

The comparison between persistent and non-persistent dimethyl phosphate esters shows that they produce different biochemical lesions after sublethal intravenous injection. Thus LD_{50} doses, although equilethal by definition, cannot be regarded as equitoxic. There is no doubt that the ability to tolerate a further dose would be different in surviving animals if in one case inhibition were rapidly reversed and in the other prolonged for days. When the toxicity of such compounds is compared, it is not enough to determine the LD_{50} dosages, but the persistence of the inhibitor in the body must be investigated. The duration of symptoms after a single sublethal intravenous injection of dimethyl phosphate esters gives some indication of the persistence of the inhibitor (see Table 1).

The non-persistent inhibitor, Me-E 600, can be made to act as a persistent inhibitor by slow infusion. When injected for several hours into the rat's jugular vein, conversion into the second irreversible stage of inhibition took place. A decrease in the rate of infusion was followed by a remarkable increase in tolerance of inhibitor, with a parallel increase in production of irreversibly inhibited enzyme. Oral and skin absorption in certain circumstances may be analogous to slow infusion. In such cases the enzyme inactivated will be mainly inhibited irreversibly, and the animal will be more susceptible to further exposure than after intravenous injection or administration via the lungs, where absorption is also very rapid. Thus although the LD_{50} is much higher when the compound is absorbed slowly, the biochemical lesion is more serious. This conclusion applies to all dimethyl phosphate inhibitors; prolonged exposure of any kind will lead to irreversible inhibition.

Any assumption that dimethyl phosphate esters always produce a transient effect, because the cholinesterase inhibited by them is rapidly reactivated, is not justified, for two reasons. Some persist. Others, although transient after single injection, produce the same durable lesion by prolonged aministration, for example by feeding. In fact the lesion produced is more permanent than that produced by a transient diethyl phosphate ester, as can be seen by comparing Figs. 1*d–f* with Fig. 1*c*. The inactivated enzyme in the first case is irreversibly inhibited, and gain in activity is probably achieved by synthesis of fresh enzyme; in the second case the enzyme is inactivated reversibly, although the reversal is slow.

We conclude that both the degree of the persistence of the inhibitor in the body and the rate at which the inhibitor is administered will decide to what extent inhibition by dimethyl phosphate esters *in vivo* will pass from the reversible to the irreversible stage.

SUMMARY

1. Marked differences in the duration of symptoms were observed when sublethal single doses of different dimethyl phosphate esters were injected intravenously into rats. The compounds inducing prolonged symptoms (*OO*-dimethyl *S*-ethylthioethyl phosphorothiolate, *OO*-dimethyl *S*-ethylsulphinylethyl phosphorothiolate and *OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate) either persisted themselves, or produced an inhibitor which persisted for several hours in the blood. Those compounds inducing symptoms of short duration (*OO*-dimethyl *S*-ethylsulphonioethylmethyl phosphorothiolate and dimethyl *p*-nitrophenylphosphate) showed short persistence in the blood.

2. *In vivo* the rate of return of brain and erythrocyte cholinesterase activity differed markedly between the two groups of inhibitors. The persistent inhibitors produced irreversible inhibition of practically all of the inactivated enzyme, whereas most of the enzyme inhibited by the non-persistent inhibitors recovered its activity rapidly.

3. There was no distinction between the persistent and non-persistent inhibitors when tested *in vitro* for the return of cholinesterase activity by Aldridge's method. Return of activity took place at the same high rate in all instances, with a half-life of about 1·3 hr.

4. When the non-persistent inhibitor, Me-E 600, was made persistent by slow infusion for several hours into the rat's jugular vein, conversion into the second irreversible stage of inhibition took place. Decrease in the rate of infusion was followed by a remarkable increase in tolerance of inhibitor, with a parallel increase in production of irreversibly inhibited cholinesterase.

5. Because the conversion from the reversible into the irreversible stage occurs rapidly *in vivo*, it is concluded that both the persistence of the inhibitor and the route, or speed, or both, by which the dimethyl phosphate esters are administered will decide the extent to which this conversion will take place in the animal.

We thank Dr J. M. Barnes and Dr W. N. Aldridge of this Unit for their interest in our work, and Mr C. R. Kennedy and Mr D. E. A. Groom for technical assistance. We wish also to thank Dr A. Church and Dr J. W. Ridge of the Ophthalmological Research Unit, Medical Research Council, Institute of Ophthalmology, Judd Street, London, W.C. 1, for supplying details of their constant-injection apparatus.

REFERENCES

Aldridge, W. N. (1953*a*). *Biochem. J.* **54**, 442.
Aldridge, W. N. (1953*b*). *Biochem. J.* **53**, 117.
Church, A. & Ridge, J. W. (1955). *J. Physiol.* **127**, 43*P*.
Davies, D. R. & Green, A. L. (1956). *Biochem. J.* **63**, 529.
Davison, A. N. (1955). *Biochem. J.* **60**, 339.
Heath, D. F. & Vandekar, M. (1957). *Biochem. J.* **67**, 187.
Hobbiger, F. (1956). *Brit. J. Pharmacol.* **11**, 295.
March, R. B., Metcalf, R. L., Fukuto, T. R. & Maxon, M. G. (1955). *J. econ. Ent.* **48**, 355–363.
Vandekar, M. (1957). *Biochem. J.* **65**, 1P.
Wilson, I. B. (1955). *Disc. Faraday Soc.* **20**, 119.

Aspects of Vitamin A Deficiency in the Rat

By F. W. HEATON, J. S. LOWE and R. A. MORTON
Department of Biochemistry, University of Liverpool

(*Received* 4 *January* 1957)

McCollum & Davis (1913) and Osborne & Mendel (1913*a*) independently proved that fat-soluble material was indispensable in the diet of the growing rat. Since that time great progress has been made in many aspects of the study of vitamin A, but only in respect of scotopic vision is much known about its mode of action. Research into the systemic mode of action is halted for want of a useful clue.

Lowe, Morton & Harrison (1953), using chromatography and ultraviolet-absorption spectroscopy, detected a new constituent in the unsaponifiable fraction from the livers of vitamin A-deficient rats. The substance, designated SC pending elucidation of its nature, is characterized by absorption maxima at 275 and 332 mμ, with sharp inflexions at 233 and 283 mμ (solvent, *cyclo*hexane).

If SC appears as a direct result of vitamin A deficiency its chemical nature should throw light on processes regulated by the vitamin. If, however, SC occurs as an indirect consequence of vitamin A deprivation its immediate relevance will be less obvious.

The present paper is concerned with comparing (qualitatively and quantitatively) the unsaponifiable fractions of certain tissues from vitamin A-deficient rats and from similar rats given an adequate supplement of vitamin A. (For a preliminary report, see Heaton, Lowe & Morton, 1955.) These experiments lead to conclusions which are, in a limited sense, quite clear-cut, but experiments on chicks and on adult hens and cockerels (Lowe, Morton, Cunningham & Vernon, 1957) showed that

the work on the rat may have uncovered a special manifestation of vitamin A deficiency rather than a general one. At the present stage the need is for exploration and perhaps for establishing that a new approach is possible and worth while.

MATERIALS AND METHODS

Normal rat tissues were obtained from adult animals of the stock colony reared on animal diet no. 4 (British Extracting Co. Ltd., Bromborough Port, Cheshire) supplemented by bread and milk.

Vitamin A-deficient rats were produced by feeding the following diet to weanling animals: casein, 18% (either 'low vitamin content' from Genatosan Ltd. or 'fat-free and vitamin-free' from British Drug Houses Ltd.); rice starch, 65%; arachis oil, 5%; Marmite, 8%; salt mixture (Osborne & Mendel, 1913*b*), 4%. At first each rat was given approximately 8 g. of food/day; after 3 weeks the allowance was increased to approx. 10 g./day, but towards the end of the experiment the rats did not eat all the food provided. Water was supplied without restriction throughout the experiment.

When after 6–7 weeks on the diet the rats exhibited xerophthalmia and severe loss of weight they were anaesthetized and killed by cardiac puncture. A syringe was used and blood collected (Glover, Goodwin & Morton, 1947).

The control rats were similar weanling animals given the same amount of the vitamin A-deficient diet as the deficient rats and, in addition, a supplement of 20 i.u. of vitamin A/day, administered orally in the form of cod-liver oil of a known potency diluted with arachis oil until the dose was contained in 0·1 ml. of the mixture. After a similar period of 6–7 weeks on the control diet, these rats were anaesthetized, killed by cardiac puncture and dissected.

Saponification and extraction of tissues. Saponification was effected by adding to the moist tissue 60% (w/v) potassium hydroxide (0·5 ml./g. of tissue) and the mixture was heated on a water bath until it became homogeneous. Ethanol (2 vol.) was then added and boiling continued under reflux for 30–45 min. For livers, kidneys, small intestines and submaxillary glands, boiling for 30–45 min. sufficed, but for bladders, vaginas, testes and the skin from around the eyes at least 2–3 hr. refluxing was necessary for complete hydrolysis of tissue. Water was then added and the mixture extracted five or six times with diethyl ether (freshly distilled over reduced iron). The combined ether extracts were washed with water until free from alkali, dried over anhydrous sodium sulphate, filtered through a sintered-glass funnel (G 3) and evaporated to dryness under nitrogen.

Chromatography. Chromatographic separations were carried out on alumina (Grade O, P. Spence and Co. Ltd., Widnes) weakened with water to Brockmann Grade 3. Experience showed that with some batches of alumina it was necessary to wash the column with 100–150 ml. of light petroleum to remove an organic contaminant (with ultraviolet absorption showing inflexions at 235 and 260 mμ). This precaution was therefore taken with all batches. The material to be chromatographed was dissolved in the minimum volume of light petroleum and poured on the column. The chromatogram was developed at first with the same solvent and later with mixed solvents consisting of light petroleum to which had been added certain percentages (v/v) of ether. Eluates were usually examined by the Liebermann–Burchard test or the antimony trichloride test, or both, fractions being cut according to the colours they gave by these procedures. The diethyl ether and the light petroleum (A.R., b.p. 40–60°) used in chromatographic fractionations were dried over sodium wire and redistilled before use, the ether being redistilled over reduced iron. Fractions were taken to dryness under nitrogen and examined spectroscopically.

The total sterol was estimated by weighing the fractions which gave positive reactions in the Liebermann–Burchard test and the 7-dehydro steroid was estimated spectrophotometrically, using a three-point correction procedure described by Glover, Glover & Morton (1952). The absorption curves characteristic of the 7-dehydro-steroid-containing fractions obtained from kidneys did not show the individual peaks (272, 282, 293·5 mμ) clearly enough to permit the use of the above technique, and a correction was made by interpolating a curve for irrelevant absorption in the region 260–300 mμ. By subtracting the irrelevant absorption at 282 mμ it was possible to correct the gross reading and arrive at an approximate intensity for the 7-dehydro steroid contribution.

RESULTS

Altogether 212 weanling rats were placed on the vitamin A-deficient diet, but fourteen died between 5.30 p.m. and 9 a.m. during the course of depletion. These animals were discarded, as it was thought that autolysis occurring over an uncertain period might possibly bring in undesirable complications.

In contrast with reports in the literature that infection of the submaxillary glands often accompanies vitamin A deficiency (e.g. Green & Mellanby, 1928) the glands from only three of the 198 xerophthalmic rats were found to contain pockets of pus visible to the naked eye.

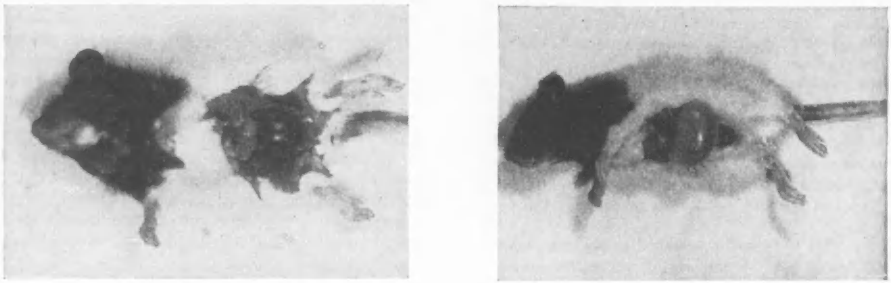
The livers from ten of the worst-affected rats (i.e. those exhibiting the greatest loss of weight or the most severe external eye lesions) appeared abnormal. These livers were distinctly yellow and had small surface nodules. Histological examination showed heavy infiltration with diffusely distributed fat, with a tendency for the centrilobular areas to be less affected than the mid-zones and periportal areas. The lipid was not doubly refractile and seemed to be neutral fat; it occurred in the parenchymal cells and not in the Kupffer cells.

The kidneys from several of the rats with fatty livers displayed hard, greyish green superficial nodules, and kidneys from a few xerophthalmic rats with apparently normal livers were likewise affected. Histological examination revealed, as with the livers, infiltration of neutral fat. The abnormal deposition occurred in the mitochondrial zone of the cells and was restricted to the proximal convoluted tubules.

Some of the deficient rats were found to have a grossly swollen stomach or bladder, the distension appearing to be the result of a blockage at the pyloric sphincter or the exit of the urethra. Sixteen stomachs and thirty-one bladders similar to those shown in Fig. 1 were encountered in the 198 xerophthalmic rats examined. No abnormal livers, kidneys, stomachs or bladders were found in the forty-three control rats and the fifty-one stock colony rats examined in the course of this work.

The gross unsaponifiable materials obtained from the various organs were examined spectroscopically, the results being summarized in Table 1.

Each batch of unsaponifiable material was chromatographed on alumina: a typical separation obtained with the unsaponifiable material from the livers of vitamin A-deficient rats is shown in Table 2. Attention will be focused on fractions 3–7, as these contained substances recognizable by their characteristic absorption spectra. Both fractions 3 and 4



(*a*) (*b*)

Fig. 1. Vitamin A-deficient rats showing (*a*) greatly distended urinary bladder, (*b*) greatly distended stomach.

Table 1. *Comparison of organs from stock colony, control and vitamin A-deficient rats*

Organ or tissue	Group	No. of rats	Mean wt. of organ or tissue (g.)	Mean vitamin A content (i.u./g.)	Mean % unsaponifiable	Principal absorption peak in *cyclo*hexane (mμ)	$E^{1\%}_{1cm.}$ calc. (on unsaponifiable matter)
Liver	Stock colony	28	9·82	435	0·322	327	77·6
	Control	43	5·37	11·6	0·412	272	9·2
	Vitamin A-deficient	185	2·77	—	0·499	273–274	16·7
Skin from around eyes	Stock colony	33	0·167	—	2·10	271	3·34
	Control	43	0·072	—	3·06	270	2·17
	Vitamin A-deficient	190	0·077	—	2·49	270	2·29
Kidney	Stock colony	51	1·96	3·2	0·568	273	3·42
	Control	43	1·12	24·0	0·615	274	3·89
	Vitamin A-deficient	150	0·82	—	0·609	272	3·69
Submaxillary gland	Stock colony	33	0·539	—	0·536	272	2·56
	Control	43	0·460	—	0·437	272	2·82
	Vitamin A-deficient	190	0·193	—	0·690	272	3·28
Small intestine	Stock colony	16	9·82	0·15	0·264	272	4·73
	Control	43	2·74	—	0·528	272	2·85
	Vitamin A-deficient	109	3·54	—	0·309	272	3·58
Stomach	Stock colony	8	1·97	—	0·532	282	10·3
	Vitamin A-deficient	27	0·962	—	0·484	282	10·6
Bladder	Stock colony	39	0·135	—	0·641	282	7·36
	Control	43	0·0670	—	0·774	282	8·25
	Vitamin A-deficient	195	0·0470	—	0·472	282	14·9
Vagina	Stock colony	21	0·312	—	0·525	282	6·60
	Control	21	0·137	—	1·12	282	5·23
	Vitamin A-deficient	113	0·075	—	2·08	282	6·97
Testis	Stock colony	11	2·46	—	0·421	270	1·81
	Control	22	2·05	—	0·351	270	5·57
	Vitamin A-deficient	83	0·585	—	0·571	270	3·84

— signifies none found.

Table 2. *Properties of chromatographic fractions from vitamin A-deficient rat-liver unsaponifiable matter*

Unsaponifiable material (304·1 mg.) obtained from 61·1 g. of liver from 23 rats was chromatographed on a column of 30 g. of alumina, length 9·5 cm., diameter 2·1 cm. Column washed with 100 ml. of light petroleum before use. E = diethyl ether, P = light petroleum (see text).

Fraction	Eluent	Wt. of fraction (mg.)	Absorption spectra of fractions (in *cyclo*hexane)	$E^{1\%}_{1\,cm.}$
1	200 ml. of P	34·9	Max. 260–263 mμ	13·7
2	200 ml. of 2% E/P	1·6	Inflexion 260 mμ	40·3
3	300 ml. of 4% E/P	16·9	Max. 272–273 mμ	176
			Min. 237 mμ	46·5
			Step-out 330 mμ	12·6
4	300 ml. of 6% E/P	11·0	Max. 272–274 mμ	80·0
			Min. 240 mμ	31·7
			Inflexion 330 mμ	8·04
5	200 ml. of 8% E/P	3·0	Inflexion 235 mμ	54·2
			Max. 277 mμ	27·8
			Shoulder 285 mμ	26·6
			Max. 327–330 mμ	9·0
6	200 ml. of 10% + 100 ml. of 12% E/P	12·0	Inflexion 233 mμ	107
			Max. 275 mμ	46·5
			Shoulder 283 mμ	45·0
			Max. 332 mμ	18·4
7	600 ml. of 20% E/P	153·3	Inflexion 235 mμ	8·22
			Max. 275 mμ	3·37
			Shoulder 283 mμ	3·16
			Max. 332 mμ	1·32
8	200 ml. of E	1·1	Max. 272–273 mμ	70·0
			Max. 313 mμ	30·1

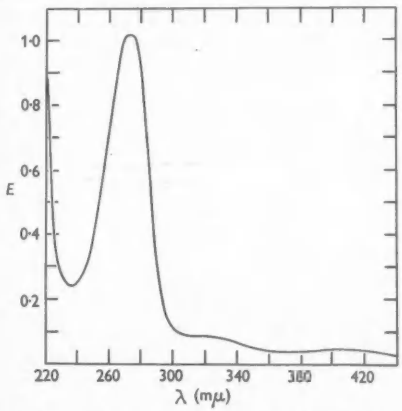


Fig. 2. Ultraviolet-absorption curve for SA in *cyclo*hexane [$E^{1\%}_{1\,cm.}$ (272 mμ), 170].

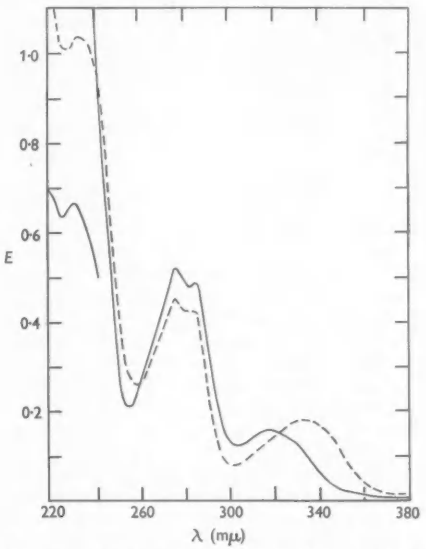


Fig. 3. Ultraviolet-absorption curves, with *cyclo*hexane as solvent, for SC (– – –); for SC acetate (——) [SC acetate gave $E^{1\%}_{1\,cm.}$ (275 mμ), 94].

were yellow and liquid. The substance in fraction 3 responsible for the characteristic absorption spectrum with $\lambda_{max.}$ 272–273 mμ, $\lambda_{min.}$ 237 mμ and a step-out at 330 mμ (*cyclo*hexane) is the material previously described by Festenstein, Heaton, Lowe & Morton (1955) and designated SA. The spectrum of fraction 4 differs from that of fraction 3 in the bathochromic displacement of the minimum and the appearance of a sharp inflexion instead of a step-out at 330 mμ (Fig. 2). The substance responsible for the sharp inflexion will be called SB. The chromophoric material in fractions 5–7 (Fig. 3), which has been designated SC, is the

substance first noted by Lowe *et al.* (1953). As can be seen from Table 2, it is very similar in polarity to cholesterol, which was the main constituent of fractions 6 and 7 (positive Liebermann–Burchard reactions).

By cutting fractions as shown in Table 2, satisfactory separations were achieved with the unsaponifiable material from most organs of vitamin A-deficient rats. Not all organs examined, however, contained SA, SB and SC. On the other hand, 7-dehydro steroid was detected in certain organs. When SC and 7-dehydro steroid occurred together, a partial separation was obtained by collecting the sterol-containing eluate in several portions; SC was eluted with the early running portion of the cholesterol, and 7-dehydro steroid accompanied the later running part of cholesterol. A complete separation of these two substances proved difficult.

When livers, kidneys and small intestines from stock colony rats were examined, the vitamin A was eluted in company with the first portion of the sterol fraction and masked the SC. In consequence, a method of separating SC had to be developed and this is shown in Table 3. The absorption spectra of SC acetate and that of the free material are compared in Fig. 3.

It can be seen from Table 1 that in both the liver and bladder, vitamin A deficiency enhances the intensity of absorption ($E^{1\%}_{1\,cm.}$) of the gross unsaponifiable material in the range 272–285mμ, indicating that the syndrome includes accumulation of substances exhibiting selective absorption. The absorption spectra of the testes gross unsaponifiable materials showed maxima in the regions 230, 270 and 280 mμ and intensities varied with conditions of saponification, suggesting possible alkali isomerization of unsaturated non-conjugated lipids.

To investigate the various changes further, the amounts of SA, SC, 7-dehydro steroid and total sterol have been estimated after chromatography, in these and other organs from vitamin A-deficient, control and stock colony rats. The results are compared in Table 4, a study of which reveals that vitamin A deficiency brings about profound changes. The arbitrary units in which SA and SC contents are expressed were obtained by multiplying $E^{1\%}_{1\,cm.}$ at 272 or 275 mμ respectively by the weight (mg.) of the chromatographic fraction. It can be seen that, although SC was obtained from the kidneys of both stock colony and vitamin A-deficient rats, it was not detected in the kidneys from the control animals. When the control rats were examined the technique of methanol precipitation and esterification (Table 3) had not been developed; so it is reasonable to assume that the large amounts of vitamin A in the kidneys of these rats (Table 1) would have masked any small amounts of SC present. Subsequent experience has shown the SC content to be very small indeed.

Table 3. *Separation of SC from organs of rats containing vitamin A*

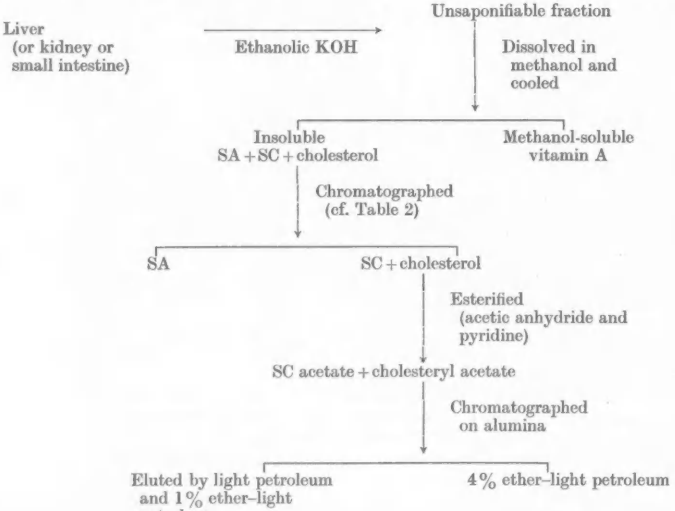


Table 4. *Effects of vitamin A deficiency on the concentrations of certain substances occurring in the unsaponifiable fractions of rat organs*

Units of absorption are expressed as $E^{1\%}_{1\,cm.}$ at 272 or 275 mμ times wt. of fraction (mg.).

Organ	Group	No. of estimations on which data are based	Mean SA (units/g.)	Mean SC (units/g.)	Mean total sterol (mg./g.)	Mean 7-dehydro steroid (mg./g.)
Liver	Stock colony	2	13·0	0·20	2·03	—
	Control	2	19·9	7·19	2·19	—
	Vitamin A-deficient	8	47·2	15·5	2·78	—
Skin from around eyes	Stock colony	1	—	—	6·90	0·0516
	Control	1	—	—	10·3	0·0371
	Vitamin A-deficient	3	—	—	11·1	0·0725
Kidney	Stock colony	4	12·1	0·516*	3·82	0·0033
	Control	2	10·6	Not detected	4·14	0·0009
	Vitamin A-deficient	4	12·5	0·453	4·08	0·0020
Submaxillary gland	Stock colony	1	3·12	—	3·83	—
	Control	1	4·70	—	3·62	—
	Vitamin A-deficient	3	6·45	—	4·68	0·0019
Small intestine	Stock colony	2	3·43	0·478	1·68	0·00374
	Control	2	2·09	0·651	2·54	0·00950
	Vitamin A-deficient	5	3·23	0·769	2·00	0·00066
Stomach	Stock colony	1	0·80	—	2·67	0·131
	Vitamin A-deficient	1	1·61	—	2·70	0·113
Bladder	Stock colony	2	—	—	1·30	0·063
	Control	1	—	—	2·29	0·076
	Vitamin A-deficient	2	—	—	2·44	0·123
Vagina	Stock colony	1	—	—	1·58	0·061
	Control	1	—	—	2·73	0·028
	Vitamin A-deficient	3	—	—	7·35	0·157
Testis	Stock colony	2	—	—	1·84	—
	Control	2	—	—	1·78	—
	Vitamin A-deficient	4	—	—	3·14	—

— signifies substance sought but not found.
* SC estimated on one batch of kidneys (25 rats) only.

DISCUSSION

Although the occurrence of fat-infiltrated livers was unexpected, it is not the first time that it has been encountered in vitamin A-deficient rats (cf. Monaghan, 1932). However, the fatty kidneys have not previously been reported in avitaminosis A, so far as the authors are aware. Similarly a photograph of a large stomach has been published by Mayer & Krehl (1948), but the distended bladders do not appear to have been previously recorded. Neither the stomachs nor the bladders were examined histologically, but it would appear that sloughing of hyperkeratotic epithelium from the lining of the organ might account for the abnormalities.

Table 4 shows that the concentrations of SA, SC, 7-dehydro steroid and total sterol are all affected by vitamin A deficiency, but the effects vary from one organ or tissue to another. In the livers, submaxillary glands and stomachs, a rise in the content of SA occurs during deficiency; on the other hand, few changes in the amounts present in the kidney and small intestine were noted. The most prominent change in SC concentration occurs in the liver, where vitamin A deficiency has the effect of causing a large (77-fold) rise. Compared with this, the rise in SC concentration in the small intestine is small. The rise in total sterol is most marked in the vagina and testis, and the submaxillary gland, urinary bladder and vagina exhibit the greatest increases in the concentration of 7-dehydro steroid. In marked contrast is the amount of 7-dehydro steroid found in the small intestine of vitamin A-deficient rats which decreases to about one-sixth of that found in the stock colony rats.

Different tissues have varying degrees of sensitivity towards the accumulation of these substances. With the liver, there is a rise in both SA and SC during vitamin A deficiency, but the concentration of SC is more sensitive to the depletion process than that of SA, which shows a marked increase only near the time when the vitamin A reserves are quite exhausted. A similar effect occurs in the small

intestine, where SC increases slightly during vitamin A deficiency, although the amounts of SA appear to be unaffected.

SA, SC and 7-dehydro steroid could be either normal metabolic intermediates or abnormal products formed as a result of the vitamin deficiency. With the exception of the submaxillary glands (in which 7-dehydro steroid was found only in the deficient animals), these substances have always been detected, though generally in lower concentrations, in the corresponding organs from rats containing vitamin A, thereby indicating that the substances are more likely to be normal metabolic intermediates which accumulate in the deficiency state rather than wholly aberrant products of a deranged metabolism.

Possibly SA and SC are intermediates in different metabolic pathways; the extent to which they accumulate will then depend on the equilibria of the enzymic reactions in the two processes. Alternatively, SA and SC may both be intermediates in the same metabolic pathway. If so, it seems likely that SC is most closely related to the substance whose metabolism is blocked during vitamin A deficiency since, as already pointed out, it is more sensitive in its response to deficiency than SA.

The very selective distribution of 7-dehydro steroid is noteworthy. Originally this substance was regarded principally as a constituent of skin, but Glover *et al.* (1952) showed that it could be formed by desaturation of ingested cholesterol in the small intestine of the guinea pig. The concentrations of this substance in the bladder and vagina of stock colony rats are shown in Table 4 to be greater than in the small intestine and the skin around the eyes of similar rats.

Ward & Moore (1953) have examined the sex organs of the rat and have detected high concentrations of 7-dehydro steroid in the caput epididymis, corpus epididymis and preputial glands of male rats and in the bulbi vestibuli of female rats. They could find no such material in the testes (thus supporting the present results), cauda epididymis, prostates, seminal vesicles or coagulating glands. The occurrence of 7-dehydro steroid at particular sites in the urinogenital tract suggests that it is formed locally and is connected with sexual processes. Morton & Rosen (1949) found the ovaries of pre-hibernation frogs to be yet another specialized locus of 7-dehydro steroid formation. Feyel Cabanes (1949) claims that in rabbits castrated after fertilization the administration of 7-dehydrocholesterol reduces the dose of progesterone required to maintain gestation. The concentration of 7-dehydro steroid in the stomach exceeds that found in any other organ during the present investigation and suggests that the stomach is an important site of sterol metabolism.

The increased concentrations of 7-dehydro steroid observed in the eye tissue, bladder and vagina during vitamin A deficiency are not easy to reconcile with the more spectacular decrease in the concentration of this substance in the small intestine, but appear to indicate that the sterol metabolism of the intestine is quite different from that of the other organs examined. The accumulation of 7-dehydro steroid in the submaxillary gland and vagina is accompanied by the accumulation of spectroscopically transparent sterol, but, in the bladder, vitamin A deficiency appears to exert a much more specific effect on the formation or accumulation of 7-dehydro steroid.

These results indicate that vitamin A is essential for the continuance of certain normal metabolic reactions, but they give no indication of the mode of action. The most obvious suggestion is that it exerts a coenzymic action. From our present limited knowledge of the reactions of the vitamin *in vivo* it appears that oxidation to retinene is the only one which it undergoes readily, suggesting that if vitamin A has a coenzymic function it may be involved in oxidative or reductive reactions such as those of the system vitamin A $\rightleftharpoons$ retinene. The substances whose accumulation has been observed during vitamin A deficiency are unsaturated and hence would appear to be susceptible to either reduction or further oxidation.

SUMMARY

1. The unsaponifiable matter from various organs of vitamin A-deficient, control and stock colony rats has been examined by chromatographic and spectroscopic techniques.

2. Attention has been focused on the spectroscopically recognizable constituents designated SA and SC, together with total sterol and 7-dehydro steroid; the concentration of all these substances has been shown to be affected by vitamin A deficiency.

3. In livers and submaxillary glands deficiency causes a rise in the level of SA; livers also exhibit a marked concentration of SC and there is a much smaller accumulation of this substance in the small intestine. The bladder, submaxillary gland and vagina contain an increased concentration of 7-dehydro steroid, and in the latter two organs and the testes there is accumulation of spectroscopically transparent sterol; the small intestine exhibits a marked fall in 7-dehydro steroid concentration.

4. These results are discussed in relation to the systemic mode of action of vitamin A.

We wish to thank Dr J. C. Davies of the Department of Pathology, University of Liverpool, for carrying out the histological examination of liver and kidneys.

This work was done during the tenure of an Agricultural Research Council Scholarship (F.W.H.) and an I.C.I. Fellowship (J.S.L.).

Financial assistance from the Nuffield Foundation is gratefully acknowledged.

REFERENCES

Festenstein, G. N., Heaton, F. W., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **59**, 558.
Feyel Cabanes, T. (1949). *C.R. Soc. Biol., Paris*, **143**, 1454.
Glover, M., Glover, J. & Morton, R. A. (1952). *Biochem. J.* **51**, 1.
Glover, J., Goodwin, T. W. & Morton, R. A. (1947). *Biochem. J.* **41**, 97.
Green, H. N. & Mellanby, E. (1928). *Brit. med. J.* **2**, 691.
Heaton, F. W., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **60**, xviii.
Lowe, J. S., Morton, R. A., Cunningham, N. F. & Vernon, J. (1957). *Biochem. J.* **67**, 215.
Lowe, J. S., Morton, R. A. & Harrison, R. G. (1953). *Nature, Lond.*, **172**, 716.
McCollum, E. V. & Davis, M. (1913). *J. biol. Chem.* **15**, 167.
Mayer, J. & Krehl, W. A. (1948). *J. Nutr.* **35**, 523.
Monaghan, B. R. (1932). *J. biol. Chem.* **98**, 21.
Morton, R. A. & Rosen, D. G. (1949). *Biochem. J.* **45**, 612.
Osborne, T. B. & Mendel, L. B. (1913*a*). *J. biol. Chem.* **15**, 311.
Osborne, T. B. & Mendel, L. B. (1913*b*). *J. biol. Chem.* **15**, 317.
Ward, R. J. & Moore, T. (1953). *Biochem. J.* **55**, 295.

Vitamin A Deficiency in the Domestic Fowl

By J. S. LOWE, R. A. MORTON, N. F. CUNNINGHAM and J. VERNON
Department of Biochemistry, University of Liverpool

(*Received* 4 *January* 1957)

The observation that vitamin A-deficient rats accumulate two substances, SA and SC, in the liver (Lowe, Morton & Harrison, 1953; Heaton, Lowe & Morton, 1955, 1957) makes it necessary to ascertain by experiment whether the phenomenon is shown by other species as part of the deficiency syndrome.

The present paper describes experiments in which (*a*) very young chicks were placed on a vitamin A-deficient diet and compared with controls, and (*b*) fowls 16 weeks old (equal numbers of cockerels and pullets) were divided into two groups, one of which was given vitamin A in known amount as a supplement to the diet which in the other group resulted in avitaminosis A after about 8 weeks.

EXPERIMENTAL

Young chickens

Males of the Light Sussex breed were used. In a trial run they were a week old when received and put on the experimental diet. In the main experiment 1-day-old birds were given a commercial 'starter' mash for 1 week and then were put on the special diets.

Housing. The chickens were housed in an oval-shaped run with sheet-metal sides 3 ft. high. The concrete floor was covered to a depth of 4 in. with peat and the run was closed at the top with $\frac{1}{2}$ in. mesh wire netting. The temperature just above the peat was regulated by means of an infrared lamp (150 w) suspended at a variable height over the run. The distance from the lamp to the chickens was increased from week to week so that the temperature was lowered from 90° to 70° F by 5°/week. After 5 weeks there was no further need to use the infrared lamp as the temperature of the animal house was kept at 70° F by thermostatically controlled electric heaters.

The trial run established that the experimental diet produced avitaminosis A, and that no trace of vitamin A could be found in the livers of the chickens when they were killed.

In the main experiment fourteen male chickens were used. A vertical partition was placed across the run and two infrared lamps were placed so that each half of the run could be warmed. Seven chickens were placed in each compartment and water was provided at all times. The birds were weighed regularly.

Diet. Table 1 gives the composition of the diet supplied to the chicks. It is based on the work of J. G. Bieri (personal communication) and the salt mixture on that of Briggs, Spivey, Keresztesy & Silverman (1952). It should be noted that after 2 weeks the cerelose was replaced without detriment by sucrose. The control chickens were given vitamin A to the extent of 3 i.u./g. of diet. The amount of food supplied was 20 g. daily per chick for the first week, increased to 25 g. in the second week and so on, until in the sixth week the chickens were offered 40 g. per day each.

Older birds

Chicks of the Brown Leghorn breed which had been reared on free range were brought into the animal house at the age of 6 weeks and at first were housed three to a large cage and later in separate cages. After 10 weeks in the laboratory the birds were divided into two groups of twelve. Each group contained six males and six females.

Diets. From the age of 6–16 weeks the fowls were given diet A (Table 2). This provided vitamin A sufficient to meet the estimated normal requirement but not enough to build up a big reserve in the liver. Vitamin A was withheld from half the birds from the seventeenth week. This group

received an extra 1% of limestone flour but had a negligible intake of carotenoids and of vitamin A. Towards the end of the experiment the deprived fowls were not eating all the food offered. Water was provided freely.

At the end of the feeding trials, when all the vitamin A-deficient birds had been killed, the controls were without exception healthy, and the pullets were laying eggs regularly but the yolks were colourless. It was then decided to give the controls for a short time a normal food mixture which contained carotenoids. The fowls quickly stored xanthophylls, the pullets produced eggs with yellow yolks and xanthophylls were deposited in the usual sites in both sexes.

Table 1. *Vitamin A-deficient diet*

Components are given in the order of mixing.

Salt mixture*	71 g.
Marmite	50 g.
Vitamin solution†	20 ml.
Arachis oil	40 g.
(containing vitamin K_1	1 mg.
α-tocopheryl acetate	10 mg.
and vitamin D_3, 100 000 i.u./g., premixed)	30 mg.
Cerelose‡	600 g.
Light White Casein (Genatosan)	280 g.

* Salt mixture (Briggs *et al.* 1952): $CaCO_3$, 75 g.; K_2HPO_4, 45 g.; $Na_2HPO_4,12H_2O$, 92 g.; $Ca_3(PO_4)_2$, precipitated, 70 g.; NaCl, 44 g.; $MgSO_4,7H_2O$, 25 g.; ferric citrate, 2 g.; $MnSO_4,4H_2O$, 2·1 g.; KI, 0·2 g.; $ZnCO_3$, 0·1 g.; $CuSO_4,5H_2O$, 0·1 g. This is enough for five batches of the major components.

† Vitamin solution: thiamine hydrochloride, 32·5 mg.; riboflavin, 25 mg.; Ca pantothenate, 85 mg.; nicotinamide, 350 mg.; pyridoxine hydrochloride, 30 mg.; biotin, 1·25 mg., *p*-aminobenzoic acid, 100 mg.

‡ After 2 weeks the cerelose was replaced by sucrose.

Table 2. *Diet A*

The diet was fed in the laboratory to 24 chickens until they reached the age of 16 weeks, and thereafter was fed minus vitamin A to twelve chickens until they became deficient. The twelve controls continued to receive this diet until all the deficient group had been killed, and were then transferred for a short time to a normal ration containing carotenoids. Amounts used were 50 g./day in the seventh week, increasing by 10 g./day in each succeeding week, until in the seventeenth and subsequent weeks the amount was 120 g./day.

	%
Dried skimmed milk	2·5
Dried whey	2·5
Extracted sunflower-seed meal	5·0
Extracted soya-bean meal	5·0
Pollards	28·75
Wheat	15·0
Milo	35·0
Dried yeast	2·5
Minerals*	3·75
Vitamin D_3, 2·2 i.u./g.	
Vitamin A, 3 i.u./g.	

* NaCl, 10; Ca_2HPO_4, 45; $CaCO_3$ (limestone flour), 40; trace-element limestone, 5%.

Avitaminosis

The deficiency symptoms observed are described below. It was planned to kill the birds when deficiency had become evident; in general this aim was achieved with the older birds, but some of the young birds died suddenly, with only early signs of depletion and no interruption of growth.

Examination of tissues

After the birds had been killed and the required tissues had been dissected out, the procedure described by Heaton *et al.* (1957) was followed. The tissues (if necessary stored at −20° C until worked up) were saponified and the unsaponifiable fractions chromatographed on alumina (P. Spence and Co., Widnes; Grade O weakened with water to Brockmann Grade 3).

RESULTS

Deficiency symptoms in young chicks

In the trial experiment the earliest sign of deficiency appeared after 18 days. One bird had difficulty in standing and was easily knocked over by the others. Three days later another chick developed similar weakness which was shown in turn by all the birds. Even when squatting the birds were unsteady and liable to fall down; there was progressive weakness but little or no loss of appetite or weight. Between the first signs of unsteadiness and death there was an interval of about 6 days.

Post-mortem examinations showed that all but one of the chicks had pale mottled kidneys with deposits of urates in the tubules.

In the main experiment unsteady gait first appeared after 20 days of vitamin A deprivation, and within a week the seven chicks in the group had died or had been killed. None of the control chicks showed any abnormality. The vitamin A-deficient chicks increased in weight steadily, indeed almost as well as the controls. They showed no signs of xerophthalmia or of lesions in the trachea or oesophagus. The ataxia described was the only symptom of deficiency in the living bird, and kidney damage was the only post-mortem sign.

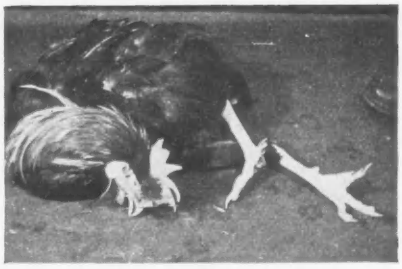
Deficiency symptoms in older fowls

The birds in this experiment were hatched on 25 April 1955, and put into cages on 7 June. In mid-July some of the cockerels were becoming definitely bow-legged but the condition was shown by X-ray examination not to be a rachitic one, nor was it due to manganese deficiency. Our veterinary colleagues were inclined to regard the cause as environmental rather than dietetic. The fowls were divided into groups on 16 August and the first sign of deficiency was recorded on 3 October. 'One of the cockerels made a gurgling sound, flapped its wings a little and fell to the bottom of the cage as though dead. After a short time, probably 30 sec.,

it seemed to recover and within 5 min. it was eating again. At first the legs seemed weak but ability to stand normally soon returned.' A few days later other cockerels were similarly affected and indeed one or more of these bouts was witnessed in each of the deprived cockerels. There always seemed to be difficulty in breathing, possibly because of blockage in the respiratory tract. Not a single pullet was seen to suffer anything resembling these fits of unconsciousness.

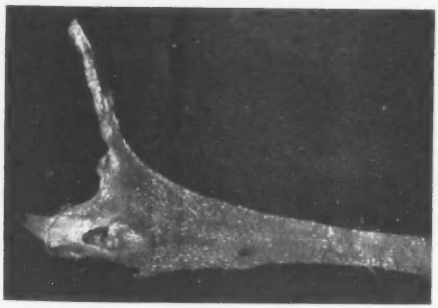
The females showed marked leg weakness and were often found sitting or leaning against the side of the cage. They would also use a wing to prop themselves upright [see Fig. 1 (*a*), (*b*)]. The females began to show this ataxia from 7 October onwards. Each pullet was killed when it became unsteady on its feet and lost interest in its food. Anorexia was a general but late symptom. (One hen was found to have 'big liver disease', a form of leukaemia. This is regarded on veterinary advice as an incident unconnected with vitamin A deficiency and irrelevant to the present inquiry.) In general, the vitamin A-deficient birds exhibited white patches in the trachea, and subsequent microscopic examination showed the presence of many squamous epithelial cells in the slime. Pustules in the oesophagus appeared as a late manifestation, mostly in males. When seen they were very marked [see Fig. 1 (*c*)]. Mucus in the nasal sinuses was frequently observed. Degeneration of the nictitating membrane with accumulation of cheesy material beneath the eyelids sometimes occurred as an almost terminal symptom of deficiency.

The male control birds showed normal comb growth and the testes were well developed, but the deprived cockerels showed very small combs and underdeveloped testes [Fig. 1 (*d*)]. Similarly, the ovaries of the deprived hens were small and underdeveloped. The control hens were laying regularly from 31 October onwards but no eggs were laid by the vitamin A-deficient pullets. On post-mortem examination most of the deficient birds had abnormal kidneys, and it seemed that the kidney lesions were more severe in the few birds which died than in those which were killed. Table 3 summarizes the symptoms observed.



(*a*)

(*b*)



(*c*)

(*d*)

Fig. 1. (*a*) Vitamin A-deficient cockerel in a temporary fit of unconsciousness. (*b*) Vitamin A-deficient pullet unable to stand. (*c*) Oesophagus from adult vitamin A-deficient cockerel. The organ was slit lengthwise and laid flat. The white spots are pustules. (*d*) The cockerel on the right was given vitamin A and shows a normal comb. The cockerel on the left was vitamin A-deficient and shows a much smaller comb.

Blood analysis for uric acid

Blood was taken from the wing veins of control and vitamin A-deficient birds just before they were killed (12 controls, 8 vitamin A-deficient birds). The uric acid content was determined by means of Folin's (1934) reagent (King, 1946). The average value was 4·5 mg./100 ml. and no significant difference was obtained between the control birds and the deficient birds for either sex.

Table 3. *Symptoms observed in fowls deprived of vitamin A*

Symptom	No. of birds affected out of 6	
	Males	Females
Loss of appetite	6	6
Bouts of unconsciousness accompanied by difficulty in breathing	6	0
Failure to stand (leg weakness)	2	5
Lesions of the external parts of the eye; degeneration of nictitating membrane	3	2
Underdeveloped sex organs	6	6
Mucus blocking nasal sinuses	3	2
Oesophageal pustules	5	3
Tracheal lesions	6	4
Kidney lesions	Most severe in birds that died, but present in all	

Examination of tissues

Tables 4 and 5 summarize for chicks and fowls respectively the weights of tissue, the percentages of unsaponifiable matter and the gross spectral absorption for the control and the deficient groups. In the case of liver tissue the rather large amounts of vitamin A shown by the control groups made chromatography essential before the amounts of other constituents could be estimated. Indeed examination of the unfractionated unsaponifiable fractions for all the tissues is mainly useful in confirming that the deprived birds were in fact very thoroughly depleted of vitamin A.

Chromatographic analyses (*Tables* 6 *and* 7). Fractionation of the liver unsaponifiable matter from the controls showed that: (i) light petroleum eluted a small fraction containing a '260 mμ'

Table 4. *Unsaponifiable matter from chicken tissues*

Description	Average wt. of organ (g.)	Unsaponifiable matter (%)	Absorption spectrum in *cyclo*hexane (mμ)	$E^{1\%}_{1\,cm.}$
(*a*) Liver				
Vitamin A-deficient (trial)	11·4	0·47	$\lambda_{max.}$ 272	4·4
			~277	4·1
			~330	0·8
Vitamin A-deficient	12·3	0·35	$\lambda_{max.}$ 272	3·6
			~276	3·5
			~330	0·7
Control	12·7	0·33	$\lambda_{max.}$ 272	8·2
			~276	8·1
			~320	3·3
			$\lambda_{max.}$ 326	3·4
(*b*) Kidney				
Deficient (trial)	3·4	0·56	$\lambda_{max.}$ 270	2·0
			~278	1·9
Deficient	4·2	0·45	~270	1·5
			~278	1·4
Control	4·7	0·27	$\lambda_{max.}$ 271	2·8
			~275	2·7
			~320	0·8
(*c*) Intestine				
Deficient (trial)	12·0	0·34	$\lambda_{max.}$ 271	2·2
			$\lambda_{max.}$ 280	2·2
Deficient	11·3	0·30	$\lambda_{max.}$ 270	2·1
			~275	1·9
			~330	0·4
Control	14·04	0·31	~263	2·6
			$\lambda_{max.}$ 270	2·9
			~280	2·7
			~293	1·6
			~330	0·7

~ = inflexion.

Table 5. *Unsaponifiable matter from fowl livers, intestines, kidneys and other tissues*

Description	Average wt. of organ (g.)	Unsaponifiable matter (%)*	Absorption spectrum in *cyclo*hexane (mμ)	$E^{1\%}_{1\,cm.}$
(*a*) Liver				
Control cockerels	41·6	0·358	$\lambda_{max.}$ 327	34·8
			$\lambda_{max.}$ 255	9·1
Control pullets	44·4	0·364	$\lambda_{max.}$ 327	21·3
			$\lambda_{max.}$ 257	7·8
Deficient cockerels	22·6	0·388	†$\lambda_{max.}$ 272	2·9
			277	—
Deficient hens	26·2	0·444	†$\lambda_{max.}$ 272	1·95
			277	1·7
(*b*) Kidney				
Control cockerels	16·7	0·27	$\lambda_{max.}$ 272	2·53
			320	1·55
Control pullets	17·5	0·32	$\lambda_{max.}$ 272	2·74
			320	0·88
Deficient cockerels	12·5	0·25	†$\lambda_{max.}$ 272	2·02
			277	1·95
			330	0·47
Deficient hens	9·6	0·42	†$\lambda_{max.}$ 272	1·34
			280	1·26
(*c*) Intestine				
Control cockerels	35·6	0·29	‡$\lambda_{max.}$ 272	2·5
			325	0·9
Control pullets	26·5	0·25	‡$\lambda_{max.}$ 272	3·1
			320	0·76
Deficient cockerels	37·6	0·33	$\lambda_{max.}$ 272	2·5
Deficient pullets	31·8	0·31	$\lambda_{max.}$ 272	1·5
			282	1·4
(*d*) Oesophagus and crop				
Control cockerels	16·9	0·25	270	1·56
			325	0·57
Control pullets	13·2	0·24	270	1·22
			340	0·32
Deficient cockerels	15·0	0·21	270	1·4
			280	1·23
Deficient pullets	15·8	0·30	270	1·25
			280	1·2
			330	0·4
(*e*) Gizzard				
Control cockerels	31·8	0·18	‡$\lambda_{max.}$ 268	2·0
			320	0·5
Control pullets	18·5	0·19	‡$\lambda_{max.}$ 270	2·7
			330	0·5
Deficient cockerels	28·3	0·24	$\lambda_{max.}$ 269	4·9
Deficient pullets	22·0	0·20	$\lambda_{max.}$ 270	1·8
(*f*) Trachea				
Control cockerels	5·1	0·27	$\lambda_{max.}$ 270	2·3
			282	2·3
			325	1·0
Control pullets	3·7	0·28	$\lambda_{max.}$ 170	1·9
			280	1·8
Deficient cockerels	5·7	0·19	$\lambda_{max.}$ 271	1·9
			281	1·8
			330	0·9
Deficient pullets	3·0	0·29	$\lambda_{max.}$ 272	2·2
			277	2·1
			340	0·7

* Based on the wt. of the organ.

† No colour with $SbCl_3$ reagent for vitamin A.

‡ Carotenoids, mainly carotenols, were also present.

substance, anhydrovitamin A and carotenoid hydrocarbon; (ii) 2% ether–light petroleum eluted further carotenoid products; (iii) 4% ether–light petroleum began to remove vitamin A which, however, in the main accompanied the cholesterol, which was eluted by 8–20% ether–light petroleum. Ether alone eluted a mixture of xanthophylls accompanied by what might have been a little tocopherol. Ethanol eluted further xanthophylls.

The bulky sterol–vitamin A fraction was acetylated and the mixed acetates were chromatographed again on alumina (Brockmann, Grade 3). The bulk

Table 6. *Comparison after chromatography of unsaponifiable fractions obtained from tissues of vitamin A-deficient chicks and controls given vitamin A*

Description	Vitamin A (i.u./g. of tissue)	SA (u./g. of tissue)	SC (u./g. of tissue)	Total sterol (mg./g. of tissue)	7-Dehydro steroid (μg./g. of tissue)	Carotene (μg./g. of tissue)	Xanthophyll (μg./g. of tissue)
(*a*) Liver							
Deficient (trial)	a	10·8	a	3·0		Not estimated	
Control	14·7	6·98	0·36	2·2	0·78	0·05	0·32
Deficient	a	5·18	0·38	2·7	0·70	a	0·21
(*b*) Kidney							
Deficient (trial)	a	1·68	a?	3·4	Trace	Very low	
Control	3·8	1·91	a?	1·9	Trace	Very low	
Deficient	a	—	a?	2·6	Trace	Very low	
(*c*) Intestine							
Deficient (trial)	a	0·52	0·16	2·0	0·6	a	Not estimated
Control	a	1·57	0·16	2·2	6·0	a	0·19
Deficient	a	2·04	0·16	2·3	0·5	a	0·04

a signifies not detected.

Table 7. *Comparison after chromatography of unsaponifiable fractions obtained from tissues of vitamin A-deficient fowls and controls given vitamin A*

Description	Vitamin A (i.u./g. of tissue)	SA (units/g. of tissue)	SC (units/g. of tissue)	Steroid (%)	'Dienone' (mg./100 g.)
(*a*) Liver*					
Control cockerels	205	6	1·1	0·25	a
Control pullets	122	9·1	0·9	0·24	a
Deficient cockerels	a	2·4	0·8	0·27	0·04
Deficient pullets	a	7·1	0·7	0·34	0·04
(*b*) Kidney*					
Control cockerels	7·8	1·2	0·7	0·18	a
Control hens	5·0	2·8	1·2	0·18	0·03
Deficient cockerels	a	1·5	0·3	0·21	a
Deficient pullets	a	1·4	0·35	0·30	0·02
(*c*) Intestine†					
Control cockerels	a	0·94	0·46	0·19	0·03
Control pullets	a	0·32	0·28	0·17	0·034
Deficient cockerels	a	0·25	a	0·21	0·023
Deficient pullets	a	0·28	a	0·24	0·019
(*d*) Oesophagus and crop					
Control cockerels	a	0·3	a	0·146	a
Control pullets	a	a	a	0·136	0·07
Deficient cockerels	a	a	a	0·16	0·09
Deficient pullets	a	a	a	0·19	a
(*e*) Gizzard					
Control cockerels	a	0·7	a	0·29	0·18
Control pullets	a	0·9	a	0·13	0·12
Deficient cockerels	a	0·3	a	0·17	0·90
Deficient pullets	a	0·6	a	0·13	0·11

a = not detected.

* Where two batches were studied, the results have been expressed as means. Because of the nature of the separations the values given for SA and SC are only approximate.

† Also contained 0·3–0·9 mg./100 g. of 7-dehydro steroid.

Table 8. *Further examination of gizzard tissues from normal healthy cockerels*

Gizzard tissue (*a*)	Wt. of tissue (g.)	Unsaponifiable matter (%)	Absorption spectrum in *cyclo*hexane (mμ)	$E^{1\%}_{1\,cm.}$	Cholesta-3:5-dien-7-one (mg./100 g. of tissue)
Lining	26·5	0·224	267	4·63	0·05
Muscle	275·0	0·21	$\lambda_{max.}$ 270	1·36	0·025
(*b*)		Percentage lipid			
Lining	35	0·24	230	19·9	—
			260	10·8	—
			320	4·8	None found
			$\lambda_{max.}$ 412	12·6	—
			$\lambda_{max.}$ 436	5·0	—
			470	2·2	None found
Muscle	79	1·97	$\lambda_{max.}$ 270	1·0	—
			280	0·9	—
			400	0·1	—

(*c*) Gizzards were separated into lining and muscle and lipid was extracted from each tissue. The residue was in each case digested with alkali and the unsaponifiable fraction extracted.

Tissue	Fraction tested	Cholesta-3:5-dien-7-one (mg./100 g. of tissue)
Gizzard lining	Unsaponifiable fraction from lipid	None found
	Unsaponifiable fraction from residue	0·03
Gizzard muscle	Unsaponifiable fraction from lipid	None found
	Unsaponifiable fraction from residue	0·01

of the product, i.e. cholesteryl acetate and vitamin A acetate, was eluted easily by 1% ether–light petroleum. Fractions eluted by more polar mixtures indicated that SC-acetate was present together with carotenoids. In order to make certain that SC was present the fractions showing $\lambda_{max.}$ 275 mμ were combined, dissolved in a little light petroleum and cooled by means of a mixture of ice and salt. An ice-cold solution of iodine in petroleum was then added with vigorous stirring. After 10 min. the mixture was filtered and excess of iodine removed from the filtrate by washing with aqueous sodium thiosulphate. The precipitate, consisting of a carotenoid–iodine adduct, was discarded. The light-petroleum solution, after washing and drying, displayed the spectrum of SC-acetate very clearly. The iodine method of removing carotenoids was first used by Kuhn & Lederer (1932).

The results (Table 7) on gizzard indicated the presence of cholesta-3:5-dien-7-one in relatively high quantity. It was decided to follow up this observation by examining the muscle wall and the lining separately (Table 8). There was less dienone in the gizzards of normal cockerels than was found in those of the birds on the experimental diet, but the substance was detected after chromatography and differentiated from SA by its selective absorption at 355 mμ in concentrated sulphuric acid. Weight for weight the lining contained twice as much dienone as muscle, but no cholestadienone could be found in the lipid from either tissue. If, however, the residue which remained after the comminuted tissue had been exhaustively extracted with hot ethanol was itself digested with alkali and refluxed in the presence of ethanol, the ether extract contained the dienone. From these experiments it would seem that cholesta-3:5-dien-7-one (or a precursor) is present in the non-lipid portion of both the linings and the cell wall. Leg muscle of fowl saponified similarly did not yield any dienone.

DISCUSSION

The earlier work on vitamin A deficiency in young chicks (Hart, Halpin & Steenbock, 1922; Emmett & Peacock, 1923; Elvehjem & Neu, 1932; Almquist & Mecchi, 1939) showed that ataxia, inco-ordination and ruffled feathers appeared after about 4 weeks without vitamin A; there was little evidence of xerophthalmia or loss of appetite. Kidneys were pale and the tubules distended by accumulations of urates. Cruickshank, Hart & Halpin (1927) suggested that as they found only slight kidney lesions in chicks expected to survive only a day or so, the accumulation of urates in renal tubules and ureters was a late and possibly somewhat indirect sequel to vitamin A depletion.

Inflammation about the eyelids has been seen in older deprived birds (Taylor, Stern, Russell & Jungherr, 1947); but it is widely agreed that the xerophthalmia, which is a reliable indicator of avitaminosis A in rats, is not seen in young chicks. On the other hand, the leg weakness and uncertain gait of depleted chicks is a good indicator and is distinct from rickets.

The keratinizing metaplasia of vitamin A deficiency (Wolbach, 1954) is sometimes shown in the mouth, larynx, pharynx and oesophageal glands of vitamin A-deficient fowls (Almquist & Mecchi, 1939), but the lesions are more marked in older birds than in young chicks. Fletcher & Rigdon (1949) recorded well-defined pathological changes in the central nervous system of vitamin A-deficient ducks, and Rigdon (1952) found conspicuous degeneration of nerve cells not attributable to mechanical pressure by deformed bone on nerve. The view (cf. Rude, Bieri & Rigdon, 1951) that in vitamin A-deficient chicks bone distortion cannot account for the neurological symptoms is not consistent with the experience of Wolbach, Bessey & Hegsted (1952) and with the work of Mellanby (1938, 1947) on mammals. Adamstone (1947), on the other hand, found in vitamin A-deficient chicks slow growth of both skeleton and central nervous system.

Lamming, Woollam & Millen (1954), however, reported in vitamin A deficiency a stenosis of the cerebral aqueduct and hydrocephalus. Woollam & Millen (1955) observed as an early sign of vitamin A deficiency in the chick a significant rise in cerebrospinal-fluid pressure.

There are neurological sequelae to vitamin A deficiency, but the nature of the primary dysfunction has yet to be established. If a serious decrease in blood plasma vitamin A level has as its primary morphological sequel a thickening of the cerebral aqueduct the resulting stenosis could raise the cerebrospinal fluid pressure. The pathology of vitamin A deficiency might then vary from one species to another, in the sense that comparative anatomy would determine which neurological manifestation would be the first to appear in the different species.

The deficiency syndrome in young chicks described on p. 216 reproduced the experience of earlier workers. Chromatography carried out on the unsaponifiable fractions from liver and kidney showed that vitamin A was absent from these organs in the deprived animals and present in the controls. SA was found in all the tissues studied (Table 6), but the differences between the controls and the deprived chicks were not appreciable. SC was detected in liver and intestine but doubtfully in kidney. The only substantial difference between the vitamin A-deficient and the control group was in respect of the 7-dehydro steroid content of the tissue of the small intestine. This fell in deficiency to one-twelfth of that shown by the controls. A similar effect was also seen in rats (Heaton *et al.* 1957).

The portion of liver unsaponifiable matter most readily eluted by non-polar or weakly polar eluents probably consists mainly of transparent hydrocarbon contaminated with a substance showing $\lambda_{max.}$ 260 mμ. The amount of this material seems to increase as a result of vitamin A deficiency both in chick and rat.

For SA and SC, however, the chick experiments and the rat experiments are quite different. In rat liver SA rises and SC rises greatly in avitaminosis A, whereas in chick liver no change occurs.

In the experiment with older birds the chicks were given a good start on free range and from the age of 6–16 weeks all the birds received 3 i.u./g. of vitamin A in their food. This amount was sufficient to meet the requirement (Williams, Lampman & Bolin, 1939; Sherwood & Fraps, 1940; Jungherr, 1943) and in the controls provided for normal growth and development, including production of eggs. No vitamin A was found in liver or kidney in the deprived birds and the reserves had been exhausted before the birds were killed or died. The bouts of unconsciousness suffered by the cockerels do not seem to have been described before and the marked sex difference is new. The gross differences in the appearance of secondary sex characteristics are important and afford strong evidence of an influence, possibly indirect, of vitamin A on sterol metabolism.

Although vitamin A deficiency was shown in the deprived cockerels and pullets, no substantial difference in respect of SA, SC, total sterol, 7-dehydro sterol and cholesta-3:5-dien-7-one could be found (Table 7) between the tissues of the control and the deficient birds. The gizzard may be an exception but this requires further study.

For the adult birds, as for the chicks, there is an unequivocal failure to reproduce the changes in SA- and SC-content shown by the rat, although these two substances are regularly found in small amounts in the organs of both chicks and older birds.

The work on the rat (Lowe *et al.* 1953; Heaton *et al.* 1957) envisaged the possibility that SA or SC (or both) accumulated because a metabolic process in which vitamin A was needed could no longer occur. The work on poultry does not support this conclusion. In the birds studied here SA and SC occur in amounts consistent with their being essential metabolites independent of vitamin A. This raises a problem concerning the origin of SA and SC in the rat, and it becomes necessary to inquire whether the microbiological situation in the alimentary tract is more sensitive in some species to vitamin A deficiency than in others.

SUMMARY

1. Young male chicks (Light Sussex breed) placed on a vitamin A-deficient diet became unsteady on their legs in the fourth week and soon afterwards died. The kidneys were pale and showed deposits of urates. There was, however, no loss of appetite or weight, no xerophthalmia or lesions in the tracea or oesophagus.

2. Older birds (Brown Leghorn breed) of both sexes were put on a vitamin A-deficient diet at 16 weeks of age. Control birds were given vitamin A as a supplement. Deficiency symptoms began to appear after 7 weeks of deprivation; the males suffered recurring brief loss of consciousness and the females found difficulty in standing. Epithelial lesions were a late symptom. Sexual development was greatly retarded in both sexes.

3. Various tissues (liver, kidney, intestine) were obtained from the young chicks and from control birds and the unsaponifiable fractions were examined by chromatographic and spectrophotometric methods. The rise in SA and SC which occurs in vitamin A-deficient rat tissue did not appear in the chick tissues.

4. Similar study of tissues (liver, kidney, intestine, gizzard, oesophagus and crop, trachea) from older birds of both sexes made vitamin A deficient revealed no real difference in respect of SA, SC, total sterol, 7-dehydrocholesterol and cholesta-3:5-dien-7-one between the controls and the deprived birds.

5. Preliminary work on gizzard muscle and lining showed the presence of cholesta-3:5-dien-7-one in both tissues.

Financial assistance from the Nuffield Foundation is gratefully acknowledged (J.V.). One of us (J.S.L.) participated in the work as holder of an I.C.I. Fellowship and another (N.F.C.) as an Agricultural Research Council Scholar.

REFERENCES

Adamstone, F. B. (1947). *Arch. Path.* **43**, 301.
Almquist, H. J. & Mecchi, E. (1939). *Poult. Sci.* **18**, 129.
Briggs, G. M., Spivey, M. R., Keresztesy, J. C. & Silverman, M. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 113.
Cruickshank, E. M., Hart, E. B. & Halpin, J. G. (1927). *Poult. Sci.* **7**, 9.
Elvehjem, C. A. & Neu, V. F. (1932). *J. biol. Chem.* **97**, 71.
Emmett, A. D. & Peacock, G. (1923). *J. biol. Chem.* **56**, 679.
Fletcher, D. E. & Rigdon, R. H. (1949). *Nutr. Rev.* **7**, 188.
Folin, O. (1934). *J. biol. Chem.* **106**, 311.
Hart, E. B., Halpin, J. G. & Steenbock, H. (1922). *J. biol. Chem.* **52**, 379.
Heaton, F. W., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **60**, xviii.
Heaton, F. W., Lowe, J. S. & Morton, R. A. (1957). *Biochem. J.* **67**, 208.
Jungherr, E. (1943). *Bull. Storrs agric. Exp. Sta.* no. 250.
King, E. J. (1946). *Micro-analysis in Medical Biochemistry.* London: Churchill.
Kuhn, R. & Lederer, E. (1932). *Ber. dtsch. chem. Ges.* **65**, 639.
Lamming, G. E., Woollam, D. E. M. & Millen, J. W. (1954). *Brit. J. Nutr.* **8**, 363.
Lowe, J. S., Morton, R. A. & Harrison, R. G. (1953). *Nature, Lond.*, **172**, 716.
Mellanby, E. (1938). *J. Physiol.* **94**, 380.
Mellanby, E. (1947). *J. Physiol.* **105**, 382.
Rigdon, R. H. (1952). *Arch. Path.* **53**, 239.
Rude, J. E., Bieri, J. G. & Rigdon, R. H. (1951). *Arch. Path.* **52**, 299.
Sherwood, R. M. & Fraps, G. S. (1940). *Bull. Tex. agric. Exp. Sta.* no. 583.
Taylor, M. W., Stern, J. E., Russell, W. C. & Jungherr, E. (1947). *Poult. Sci.* **26**, 243.
Williams, J. K., Lampman, C. E. & Bolin, D. W. (1939). *Poult. Sci.* **18**, 268.
Wolbach, S. B. (1954). *The Vitamins*, vol. 1, p. 107. Ed. by Sebrell, W. H. & Harris, R. S. New York: Academic Press.
Wolbach, S. B., Bessey, O. A. & Hegsted, D. M. (1952). *Arch. Path.* **54**, 13.
Woollam, D. H. M. & Millen, J. W. (1955). *Nature, Lond.*, **175**, 41.

Vitamin A Deficiency in the Rat, With and Without Added Dietary Cholesterol

By B. GREEN, J. S. LOWE and R. A. MORTON
Department of Biochemistry, University of Liverpool

(*Received* 4 *January* 1957)

Certain aspects of the vitamin A-deficiency syndrome in the rat have been studied by Heaton, Lowe & Morton (1955, 1957). These workers examined the unsaponifiable portions from various tissues of normal, control and vitamin A-deficient rats and drew attention to marked increases in two unidentified substances, designated SA and SC, in the livers of the vitamin A-deficient rats. Lowe, Morton & Harrison (1953) had earlier put forward, as a working hypothesis, the idea that vitamin A exerts a restraining influence on the dehydrogenation of cholesterol or steroid hormones, such restraint being lifted in vitamin A deficiency. The hypothesis that this might account for the appearance of SA

and SC was tested when Green, Lowe & Morton (1955) studied in rats the plasma and liver cholesterol levels during vitamin A deficiency. They found no significant difference between plasma cholesterol levels of the deficient and control rats at any stage during the depletion.

The present work is concerned with a study of the vitamin A-deficiency syndrome in rats subjected to the additional hazard of cholesterol feeding.

EXPERIMENTAL

The preparation of unsaponifiable extracts and the chromatographic procedures are described by Heaton *et al.* (1957).

Experiment 1

Animals. Forty weanling rats were divided into two groups (*A* and *B*) of twenty each. The aim was to match each rat in group *A* with a litter mate of the same sex and approximately the same weight in group *B*, but one female in group *A* had to be balanced by a male from another litter, so that group *A* was actually made up of eight males and twelve females and group *B* of nine males and eleven females.

Diet. The experimental diet was as follows: casein ('low vitamin content', Genatosan Ltd.) 18, maize starch 60, arachis oil 10, Marmite 8, salt mixture (Osborne & Mendel, 1913) 4 parts by weight. To each 100 g. of the diet were added α-tocopherol (3 mg.), calcium pantothenate (1 mg.) and choline chloride (80 mg.), the tocopherol being dissolved in the arachis oil before mixing the food. The rats in group *B* were also given crystalline cholesterol to the extent of 2% (w/w) of diet. The cholesterol was dissolved in the arachis oil before the final mixing of the dietary constituents.

Each rat was given 8 g. of diet/day for the first 3 weeks and 10 g./day afterwards. Water was provided without restriction.

Experiment 2

A second experiment was carried out using 20 newly weaned rats divided into two groups I and II with five males and five females in each. Both groups were placed on the vitamin A-free diet supplemented with α-tocopherol, calcium pantothenate and choline, and all the rats received the addition of 2% of cholesterol to the diet. Group I also received 100 i.u. of vitamin A every 5 days during the first 41 days and then for 9 days subsequently five rats (batch 2) received the same dose every day. When a rat in group II showed vitamin A deficiency it was killed and a group I animal was killed at the same time. The animals were dissected and the tissues stored at −20° until sufficient quantities were obtained for examination.

Preparation of tissues

The test of vitamin A deficiency was the appearance of xerophthalmia accompanied by marked loss of weight. The deficient animals were anaesthetized and killed by cardiac puncture with removal of blood by means of a syringe. The liver, small intestine and kidneys in each case were removed, washed, wiped dry, weighed and stored under ethanol in the refrigerator until saponified.

The livers and intestines in Expt. 1 were saponified in three batches from six, seven and seven animals for the two groups and the kidneys were saponified in two lots of ten for each group. In Expt. 2 the tissues were saponified in batches each from five animals.

RESULTS

Experiment 1

The deficiency symptoms appeared clearly in both groups after 5–6 weeks, although growth had slackened after 4 weeks. Of the twenty rats not given cholesterol, three showed grossly distended stomachs and three grossly distended bladders (cf. Heaton *et al.* 1957). The livers of three of these rats had yellow patches on the surface and exhibited fatty infiltration. Out of the twenty rats given cholesterol one had a distended stomach and another a distended urinary bladder; all the livers in the group were grey in colour, heavy and 'fatty'. It could not be said, however, that the rats under the double risk of vitamin A deprivation and cholesterol feeding were at any very obvious disadvantage compared with the others.

Livers. Table 1 shows how liver weights varied in the two groups as the animals were killed off, in turn, on showing signs of deficiency. In every case the absorption spectrum of the total unsaponifiable fraction showed selective absorption near 272 mμ.

Table 1. *Livers of vitamin A-deficient rats* (A) *compared with livers from cholesterol-fed vitamin A-deficient rats* (B)

Batch	No. of livers	Mean wt. (g.)	Percentage unsaponifiable matter (calc. on tissue wt.)	$E^{1\%}_{1\,cm.}$ 273 mμ (calculated on unsap.)
A_1	6	2·79	0·86	8·9
B_1	6	4·84	7·3*	0·644
A_2	7	3·0	0·78	10·6
B_2	7	5·06	6·4*	0·613
A_3	7	3·63	0·84	11·37
B_3	7	5·48	9·7*	0·513
	Average *A*	$3{\cdot}15_5$	$0{\cdot}82_5$	—
	B	5·14	7·93	—

* A second 'saponification' with ethanolic KOH was necessary to complete the hydrolysis of cholesteryl esters.

Table 2. *Comparison of unsaponifiable constituents of livers from vitamin A-deficient (group A) rats with those from livers of cholesterol-fed vitamin A-deficient (group B) rats*

	Absorption units/liver*					
	Group *A*			Group *B*		
Batch	1	2	3	1	2	3
Gross absorption	211	247	344	329	271	273
Fraction 1 (petrol eluate $\lambda_{max.}$ 260 mμ)	27	34	40	40	31·6	59
Fraction containing SA	118	109	118	113	?	?†
Fractions containing SC	15·5	29	27	32·8	33	49
Cholesta-3:5-dien-7-one (mg.)	0	Trace	0	0·019	—	0·04
7-Dehydrosterol (mg.)	0	0	0	0·02	0·017	0·037

* Absorption units per liver given by the following:

$$\frac{E^{1\%}_{1\,cm.}\ \lambda_{max.}\ (\text{calc. on wt. of unsap.}) \times \text{wt. of unsap. in mg.}}{\text{no. of rats in batch}}.$$

† In order to complete the hydrolysis of cholesteryl esters it was necessary to prolong the refluxing with alkali; this resulted in destruction of much of the SA present. This loss of SA incidentally unmasked vitamin E in the group *B* livers; it was eluted by means of 6% ether–light petroleum from Brockman Grade 3 alumina.

If for each batch, the $E^{1\%}_{1\,cm.}$ 272 mμ value of the intensity of absorption is multiplied by the weight (in mg.) of unsaponifiable matter obtained and divided by the number of rats in the batch, the result is a measure of the intensity of absorption per liver. The average figure was 270 for the group *A* rats and 290 for the group *B* rats. This means that, despite the great increase in liver size which accompanied cholesterol ingestion, the total amount of selectively absorbing material remained substantially unchanged.

Table 2 summarizes the results of six chromatographic separations on three batches from groups *A* and *B*. Within the limits of experimental error the two groups do not differ in respect of (*a*) the material eluted by light petroleum and (*b*) SA, and SC. The livers from the cholesterol-fed group contained small but definite amounts of cholesta-3:5-dien-7-one (eluted by 4% ether–light petroleum, $\lambda_{max.}$ 280 mμ in *cyclo*hexane and $\lambda_{max.}$ 355 mμ in conc. H_2SO_4) which was not detected in the corresponding fraction from the livers of the other group.

The main cholesterol fraction from the group *B* rats (eluted by means of 20% ether–light petroleum) exhibited weakly the characteristic absorption of the 5:7-diene chromophore (7-dehydrocholesterol). This absorption was not detected in the cholesterol from the group *A* rats.

The liver tissue from the vitamin A-deficient rats was readily hydrolysed by alkali and the unsaponifiable material was obtained easily. Liver tissue from cholesterol-fed rats contains a good deal of esterified sterol which is only saponified with difficulty. The substance SA is, however, destroyed if the refluxing with ethanolic KOH is continued long enough to complete the hydrolysis of the cholesteryl esters. The cholesteryl esters are highly transparent to ultraviolet light, and if there had been any significant rise in SA or SC in group *B* as compared with group *A* it would have been detected even if the 'unsaponifiable' matter from the former still contained sterol esters. In Table 2 the ? sign reflects the failure to determine SA accurately after the rather prolonged heating with alkali. The disappearance of SA, however, had the incidental advantage of permitting α-tocopherol to appear in all three group *B* batches as a fraction eluted from Brockmann Grade 3 alumina by means of 6% ether–light petroleum, i.e. immediately before SC, which begins to be eluted with 8% ether–light petroleum. In the fractions obtained from group *A* livers the tocopherol tends to accompany SA and SC and to distort the absorption curves.

Kidneys. Table 3 indicates no real difference between groups *A* and *B* in respect of kidney weights, percentage unsaponifiable matter or ultraviolet absorption.

The chromatographic separations indicated the presence of very small amounts of cholesta-3:5-dien-7-one. Each pair of kidneys contained on the average 0·165 mg. of SA and 0·03 mg. of SC. In every separation, 20% ether–light petroleum eluted a material with two maxima, at 261 and 270 mμ respectively. This fraction has been shown in other work (Mervyn, 1956) to be α-tocopherylquinone. It is interesting that α-tocopherol itself could not be detected in these vitamin A-deficient kidneys, but 0·012 mg. of the quinone was found per pair. The quinone is, however, much more strongly absorbing than tocopherol so that minute amounts of the latter might have escaped detection.

Intestines. Table 4 shows that the rats of groups *A* and *B* did not differ appreciably in respect of weights of intestine, unsaponifiable matter or

intensity of ultraviolet absorption. Six chromatographic separations were carried out on Brockman Grade 3 alumina.

Light petroleum eluted a small amount of material (about 13 mg. altogether) showing a weak maximum ($E^{1\%}_{1\,cm.}$ approx. 25) at 258–260 mμ. Cholesta-3:5-dien-7-one was eluted by means of 2% ether–light petroleum, but the total amount in all the rat intestines probably did not exceed 0·5 mg. SA was eluted by means of 4% ether–light petroleum and an approximate estimate for the amount present was 3 mg. for the forty rat intestines. α-Tocopherol was clearly seen in some but not all of the separations; when found it was eluted by means of 6% ether–light petroleum, i.e. between SA and SC. The total amount of SC was about 1 mg. After SC, with 12–20% ether–light petroleum a substance was eluted with two peaks 260 and 270 mμ which are characteristic of α-tocopherylquinone. In only one batch did the sterol fraction show clearly the 271, 282, 293 mμ group of bands due to the 7-dehydro steroid structure.

Experiment 2

In this experiment all the animals received 2% of cholesterol in the diet and half the animals were given prophylactic doses of vitamin A (group I). The vitamin A-deficient animals (group II) showed

Table 3. *Kidneys of vitamin A-deficient rats* (A) *compared with kidneys from cholesterol-fed vitamin A-deficient rats* (B)

Batch	No. of kidneys (pairs)	Mean wt./pair (g.)	Percentage unsaponifiable matter (on tissue wt.)	$E^{1\%}_{1\,cm.}$ (270–272 mμ) (*cyclo*hexane) calc. on unsaponifiable matter
A_1	10	0·911	0·70	3·25
B_1	10	0·971	0·88	2·91
A_2	10	0·975	0·84	4·12
B_2	10	0·942	0·86	3·95
Means *A*		0·943	0·77	3·68
B		0·956	0·87	3·43

Table 4. *Intestines of vitamin A-deficient rats* (A) *compared with intestines from cholesterol-fed vitamin A-deficient rats* (B)

Batch	No. of intestines	Mean wt. (g.)	Percentage unsaponifiable matter (on tissue wt.)	$E^{1\%}_{1\,cm.}$ (270–273 mμ) (*cyclo*hexane) calc. on unsaponifiable matter
A_1	6	2·62	0·38	4·6
B_1	6	2·65	0·50	3·44
A_2	7	3·35	0·61	4·27
B_2	7	4·28	0·57	3·67
A_3	7	4·30	0·46	2·47
B_3	7	3·86	0·64	2·68
Means *A*		3·42	0·49	3·78
B		3·59	0·59	3·36

Table 5. *Comparison of livers from cholesterol-fed vitamin A-deficient (group II) rats with cholesterol-fed rats given prophylactic doses of vitamin A (group I)*

Batch	No. of livers	Mean wt. of livers (g.)	Total wt. of unsaponifiable matter (g.)	Percentage of unsaponifiable matter	$E^{1\%}_{1\,cm.}$ (270 mμ) (*cyclo*hexane) calc. on unsaponifiable matter	Vitamin A (i.u./g. of liver)
I_1	5	6·68	1·78	5·35	0·425	4·7
II_1	5	5·83	1·72	5·88	0·79	0
I_2	5	7·51	2·91	7·75	0·383	19·0
II_2	5	5·53	1·93	7·12	0·90	0
		Group I	4·69 (total)	6·55 (mean)		
		Group II	3·65 (total)	6·50 (mean)		

xerophthalmia, loss of weight, four had distended stomachs and one a distended bladder. The livers in both groups were markedly fatty.

Tissues rich in cholesteryl esters, as has been pointed out, are fully saponified only after prolonged refluxing with ethanolic alkali. Nevertheless, it was found that the intensity of absorption at 270 mμ was higher by 66% in the vitamin A-deficient livers than in those from animals given protective doses. This rise occurred (Table 5), although SA may have been largely destroyed.

In Table 2 the average gross absorption units/liver in group *B* rats was 290, and in this experiment it was 310 for the group II rats as compared with 180 for the rats given prophylactic doses of vitamin A.

Chromatographic separations on the liver unsaponifiable matter from group I resulted in the detection of traces of cholesta-3:5-dien-7-one, of SA, and little else other than, of course, large amounts of cholesterol. SC was not definitely found in the group II unsaponifiable fractions, although it may have accompanied the unhydrolysed sterol esters in one batch and the 7-dehydrocholesterol in the other, more completely saponified, batch. Small quantities of vitamin E were found in the livers. Perhaps the most interesting result was that the amount of cholesta-3:5-dien-7-one was not appreciably different in the two groups, nor in fact very different from the figure of 0·034 mg./liver found for adult male stock colony rats.

These weanling rats did not show the rise in 'dienone' content of liver on feeding cholesterol previously recorded by Kantiengar & Morton (1955) for adult male rats.

The intestines of both groups I and II were studied, and chromatographic separations were carried out on the unsaponifiable fractions. Traces of dienone of SA and α-tocopherylquinone were detected in groups I and II, but no important differences emerged.

DISCUSSION

Cholesterol levels are unresponsive to hepatic vitamin A stores in young rats of both sexes (Green, *et al.* 1955) or adult females, but there is a significant effect in young and adult males of cholesterol feeding on hepatic vitamin A (Kantiengar & Morton, 1955; Green, Horner, Lowe & Morton, 1957).

The accumulation of SA and particularly of SC in the livers of vitamin A-deficient rats might well have been the result of a derangement of sterol metabolism. The subjection of rats to the double hazard of excessive cholesterol intake and vitamin A deprivation neither diminished nor enhanced the accumulation of SA and SC as compared with the effects seen in uncomplicated vitamin A deficiency. The absorbed cholesterol seems to be deposited in the liver as ester and not to be in itself a direct cause of a relevant metabolic abnormality. Similarly, vitamin A deprivation superimposed on cholesterol excess does not appreciably modify the consequences of the latter.

This work has not revealed any direct or major connexion between vitamin A metabolism and sterol metabolism. No evidence has emerged that cholesterol is a direct precursor of SA or SC.

When cholesterol-fed rats given just enough vitamin A for small storage to occur in the liver are compared with cholesterol-fed vitamin A-deficient animals it is seen that the vitaminosis again results in an increased intensity of absorption near 270 mμ. The problem of obtaining unsaponifiable fractions from cholesterol fatty livers without decomposition has not been fully solved, but it seems clear from the chromatography that the increased absorption at 270 mμ in avitaminosis is not due to cholesta-3:5-dien-7-one, nor to 7-dehydrocholesterol nor to any very marked extent to SA. It is probably due in the main to SC, but this is not quite certain.

SUMMARY

1. Weanling rats on a vitamin A-deficient diet were compared with litter mates on the same diet to which cholesterol (2%) had been added. The tissues (liver, kidney, intestine) from both groups were examined when the deficiency syndrome became manifest. SA and SC (substances recognizable by their absorption spectrum) were produced in the liver in both groups. Cholesta-3:5-dien-7-one and 7-dehydrocholesterol were found in the liver unsaponifiable fraction of the group given cholesterol. The effects of vitamin A depletion and cholesterol feeding seemed additive and largely independent. No important differences were seen in respect of ultraviolet absorption shown by unsaponifiable fractions from kidney and intestine.

2. Weanling rats on a vitamin A-deficient diet containing 2% cholesterol were compared with rats on the same diet supplemented with enough vitamin A to permit small liver storage. The animals were killed when avitaminosis A was shown in the first group. The examination of tissue unsaponifiable fractions again showed little evidence of interaction between cholesterol feeding and vitamin A deprivation.

One of us (B.G.) participated in this work as holder of an M.R.C. Scholarship and another (J.S.L.) as an I.C.I. Fellow.

Support of the work by the Nuffield Foundation is gratefully acknowledged.

REFERENCES

Green, B., Horner, A. A., Lowe, J. S. & Morton, R. A. (1957). *Biochem. J.* **67**, 235.
Green, B., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **61**, 447.
Heaton, F. W., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **60**, xviii.
Heaton, F. W., Lowe, J. S. & Morton, R. A. (1957). *Biochem. J.* **67**, 208.
Kantiengar, N. L. & Morton, R. A. (1955). *Biochem. J.* **60**, 28.
Lowe, J. S., Morton, R. A. & Harrison, R. G. (1953). *Nature, Lond.*, **172**, 716.
Mervyn, L. (1956). Ph.D. Thesis: University of Liverpool.
Osborne, T. B. & Mendel, L. B. (1913). *J. biol. Chem.* **15**, 317.

Unsaponifiable Constituents of Kidney in Various Species

By J. S. LOWE, R. A. MORTON and J. VERNON
Department of Biochemistry, University of Liverpool

(*Received* 4 *January* 1957)

Two substances, designated SA and SC respectively pending determination of their structures, have been found in unsaponifiable fractions from various organs, e.g. liver and intestine, from both vitamin A-deficient and normal rats (Heaton, Lowe & Morton, 1955, 1957). The purpose of the present investigation was to study their occurrence in normal kidneys from various species, one of the objectives being to find a convenient source of either SA or SC. In the course of the work a constituent was encountered in some species, particularly cat, which was provisionally denoted SE. It was later identified as vitamin E, probably mainly α-tocopherol. In the domestic cat, the kidney was found to differ from the other species studied in having unusually large amounts of both vitamin A and vitamin E.

The substance SA occurs in the kidneys of all eleven species studied, the concentration being of the order of 20 μg./g.; it also occurs in human kidney (Mervyn, 1956). The substance SC is almost equally widely distributed, the concentration being of the order of 5 μg./g. These estimates are based on the properties (in preparation) of the purest preparations of SA and SC so far obtained.

MATERIALS

Kidneys from pig, sheep and ox were obtained as fresh as possible from the Stanley Abattoir, Liverpool. Kidneys from three piglets suspected of suffering from vitamin A deficiency were supplied by Mr Brooksbank, Veterinary Investigation Officer. Dog kidney was obtained from the Veterinary School, University of Liverpool and from the Dogs' Home, Liverpool. Hen kidneys from normal laying pullets were supplied by J. Bibby and Sons Ltd., Liverpool. Cat kidneys were obtained (*a*) from the Department of Physiology, University of Liverpool, and (*b*) through the Royal Society for the Prevention of Cruelty to Animals (R.S.P.C.A.), Liverpool Branch. When it became necessary to obtain larger quantities of material, permission was obtained from W. Smith and Sons, Lymm, to dissect carcasses bought by them from the R.S.P.C.A. The cats were unwanted and had been taken to the Society's Branches by the public and had there been painlessly killed. The dissection was done at W. Smith and Sons' premises. The material was admittedly not all entirely fresh, but no other method of obtaining adequate supplies was possible. Rabbit kidney was obtained from laboratory animals. Two batches of mouse kidney were obtained, one through the co-operation of the Wellcome Laboratories of Tropical Medicine, London, and the second with the help of The Distillers Co. Ltd., Liverpool. Lasco Ltd., Liverpool, provided kidneys from two healthy horses killed after being injured in accidents. Guinea-pig kidneys were obtained from normal laboratory animals. Kidneys from a goat came from the Department of Veterinary Pathology, University of Liverpool; the animal had been suffering from Johne's disease, but this is not known to make the kidney abnormal.

The authors wish to record their gratitude for much help in obtaining materials and to acknowledge the courtesy extended to them by many persons acting on behalf of institutions, laboratories or industrial firms.

METHODS

Preparation of unsaponifiable matter. The kidney tissue was subjected to digestion with ethanol and KOH as follows: 60% (w/v) aqueous KOH (0·5 ml./g. of tissue) was added to moist kidney and the mixture was heated on the water bath until it became homogeneous; ethanol (1 ml./g. of tissue) was then added and the mixture was boiled under reflux for 30–45 min. After cooling, water (2 vol.) was added and the solution was extracted 5 or 6 times with ether (freshly redistilled over reduced iron). The combined ether extracts were washed free from alkali, dried over Na_2SO_4, filtered through a sintered-glass funnel and evaporated to dryness under nitrogen. Any kidneys and any unsaponifiable fractions which it was necessary to store were kept at −20°.

Chromatography. The unsaponifiable matter was chromatographed on alumina (Brockmann Grade 3) in the manner described in previous papers (cf. Heaton *et al.* 1957).

RESULTS

Table 1 indicates the number of batches studied for the different species; it shows how the percentage of unsaponifiable matter varies over a narrow range and how a rather weak absorption peak near 272 mμ was shown for almost every species. The only exception was cat kidney which exhibits a surprisingly high vitamin A content. Further tests were made on cat kidney later (see p. 233). Table 2 illustrates the chromatographic separation of unsaponifiable constituents from pig kidney. Fraction 3, eluted from alumina (Brockmann 3) by 4% ether–light petroleum, shows $\lambda_{max.}$ 272 mμ and an inflexion at 320 mμ with a very weak band near 400 mμ (solvent *cyclo*hexane), and together with fraction 4 consists very largely of SA. Fraction 6, eluted by 20% ether–light petroleum, is predominantly cholesterol with some selectively absorbing contaminants. This bulky sterol fraction was acetylated by means of acetic anhydride and pyridine and the product was rechromatographed (Table 3). The least-strongly adsorbed material contains cholesteryl acetate and a trace of vitamin A acetate. The small amount of material eluted by 4% ether–light petroleum is (by selective absorption) SC acetate mixed with transparent material, e.g. traces of cholesteryl acetate. Fraction 3 eluted by 8% ether–light petroleum is a similar mixture and contains probably some free SC as well as its acetate.

Table 4 summarizes the results obtained with kidneys from various species. In these experiments the vitamin A content of kidney from mouse, guinea pig and horse was very low but the vitamin is not likely to have been wholly absent. If the vitamin A content of a tissue is of the order 1 i.u./g. special care is needed if a quantitative estimate is to be obtained. In general, kidney contains a few i.u. of vitamin A/g., but the results with cats are quite unusual (see Table 5).

The work on sheep kidney will be reported in more detail. Four batches were examined; the percentage of unsaponifiable matter ranged from 0·33 to 0·58, the vitamin A content from 3·0 to 4·3 i.u./g. and the sterol content ranged from 0·26 to 0·43%. Each batch showed the presence of carotenoids in traces only and 7-dehydro steroid absorption was just detectable. SA and SC were detected in all cases. A substance SE, which

Table 1. *Kidney unsaponifiable matter*

Species	No. of batches	Combined wt. of tissue (g.)	Average percentage of unsaponifiable matter	Absorption spectrum of unsaponifiable matter (*cyclo*hexane)	$E^{1\%}_{1\,cm.}$ (calc. on unsaponifiable matter)
Pig	3	549	0·48	272–3	2·55
Piglets	3	55·5	0·51	~235	5·0
				~270	2·1
Sheep	4	435	0·44	272	1·6
				327	0·9
Dog	2	143	0·32	~280	3·0
				$\lambda_{max.}$ 300	2·75
				~327	3·3
Rabbit	1	33·5	0·54	272	1·69
				~280	1·55
				330	0·96
Ox	2	211	0·37	272	2·4
				330	0·97
				450	1·7
Mouse	2	42	0·42	272	5·7
				~280	5·2
Horse	2	221	0·32	272	2·26
				280	2·1
Guinea pig	1	32	0·38	272	3·24
				~320	0·83
Goat	1	86	0·37	272	1·79
				~320	0·72
Cat	5	208	0·52	327	12·7
Hen	1	35	0·36	~230	4·7
				272	2·86
				327	2·98
				450	1·14

~ = inflexion.

Table 2. *Chromatography of pig-kidney unsaponifiable matter*

Alumina (Brockmann Grade 3), 20 g. Column, 10 cm. × 1·7 cm. Wt. of unsaponifiable matter used, 0·190 g.; wt. recovered, 0·167 g.

Fraction	*Eluent	Vol. (ml.)	Wt. of solute (mg.)	Absorption spectrum (*cyclo*hexane) (mμ)	†$E^{1\%}_{1\,cm.}$
1	P	200	2·0	~230	34·7
				~255	12·4
2	2% E/P	200	1·6	~235	7·5
				~255	6·1
3	4% E/P	300	2·0	$\lambda_{max.}$ 272	91·5
				~320	9·3
				$\lambda_{max.}$ 400 (flat)	4·2
4	6% E/P	200	1·4	$\lambda_{max.}$ 273–275	65·2
				~320	6·2
5	8% E/P	200	1·8	$\lambda_{max.}$ 275	7·4
				~325	1·7
6	20% E/P	900	157·3	~235	0·91
[Liebermann–Burchard test positive]				~267	0·42
				~275	0·45
				$\lambda_{max.}$ 282	0·43
				$\lambda_{max.}$ 327	0·30
7	E	200	0·6	~230	31·0
				~270	14·6
				~320	4·6

~ = inflexion.
* In this and other tables E represents diethyl ether and P represents light petroleum.
† $E^{1\%}_{1\,cm.}$ calculated on the weight of the fraction.

Table 3. *Chromatography of the sterol-rich portion of pig-kidney unsaponifiable fraction after acetylation*

Alumina (Brockmann Grade 3), 15 g. Column, 7·8 cm. × 1·7 cm. Wt. of acetate used, 0·159 g. Wt. recovered, 0·157 g.

Fraction	Eluent	Vol. (ml.)	Wt. of solute (mg.)	Absorption spectrum (*cyclo*hexane) (mμ)	$E^{1\%}_{1\,cm.}$
1	1% E/P	350	154	~255	0·25
				~275	0·20
				~285	0·18
				$\lambda_{max.}$ 327	0·15
2	4% E/P	250	0·9	~230	53·7
				$\lambda_{max.}$ 275	23·8
				~282	21·9
				~315	8·8
3	8% E/P	300	0·6	~230	89·1
				$\lambda_{max.}$ 275	38·1
				~282	35·4
				~325	14·2
4	E	200	1·0	~230	62·5
				~255	36·8
				~280	22·9

~ = inflexion.

Table 4. *Comparison of various species in respect of certain constituents of unsaponifiable material from normal kidneys*

Results are expressed as amounts/gram of kidney tissue unless otherwise stated.

Species	Vitamin A (i.u./g.)	*Tocopherol (mg./g.)	Sterol (g./100 g.)	7-Dehydro steroid	SA† (u./g.)	SC† (u./g.)
Pig	4·9	a	0·29	Tr.	2·8	0·8
Piglets with low liver vitamin A	6·6	a	0·25	a	a	0·55
Sheep	3·6	0·0041	0·32	Tr.	1·3	0·34
Dog	11·7	0·008	0·22	a	3·0	0·30
Cat	98·5	0·025	0·28	Tr.	3·1	0·21
Rabbit	3·7	a	0·35	Tr.	3·0	0·64
Ox	1·37	a	0·29	Tr.	2·3	0·20
Mouse	a	a	0·28	a	8·6	0·74
Horse	a	a	0·29	Tr.	2·2	a
Guinea pig	a	a	0·26	Tr.	4·3	a
Goat	1·25	a	0·28	Tr.	1·9	0·54
Hen	12·7	a	0·22	Tr.	3·0	0·79

a = not detected. Tr. = trace. * If small amounts of tocopherols were present they may have been destroyed.
† Arbitrary units as defined in text (see Discussion); if $E^{1\%}_{1\,cm.}$ (272 mμ) for SA is 170, one unit = 6 μg., and if $E^{1\%}_{1\,cm.}$ (275 mμ) for SC = 90, one unit = 11·1 μg.

Table 5. *Unsaponifiable fractions from cat kidney*

Kidneys from nine cats were used. The vitamin A contents were calculated from ultraviolet absorption in the region 310–340 mμ shown by the unsaponifiable matter. The three-point correction procedure (Morton & Stubbs, 1942; British Pharmacopoeia 1953) was applied, and the results are expressed as i.u./g. of kidney, wet wt.

Expt.	Wt. of kidney tissue (g.)	Unsaponifiable matter (%)	Vitamin A content* (i.u./g.)
1	30	0·72	74
2	20	0·43	69
3	14·5	0·59	103
4	31·2	0·52	134
5	112	0·34	112

Table 6. *Chromatography of sheep-kidney unsaponifiable matter*

Wt. used, 177 mg. Alumina column, 10 cm. × 1·7 cm. Alumina, 20 g. (Brockmann 3).

Fraction	Eluent	Vol. (ml.)	Wt. of solute (mg.)	Absorption spectrum (*cyclo*hexane) (mμ)	$E^{1\%}_{1\,cm.}$	Remarks
1	P	200	1·3	~255	19	—
2	2% E/P	200	1·7	~230	11·8	—
				~260	6·8	—
				~330	1·7	—
				~425	1·1	Carotenoid
				$\lambda_{max.}$ 450	1·3	—
				~475	1·0	—
3	4% E/P	200	0·2 (?)	$\lambda_{max.}$ 272	164	SA
				~315	24·5	—
4	6% E/P	200	0·2 (?)	$\lambda_{max.}$ 273	160	SA
				~300	39	—
5	8% E/P	200	1·9	$\lambda_{max.}$ 290	6·6	Vitamin E
				~295	6·2	—
6	12% E/P	100	0·1 (?)	~280	44	—
7	20% E/P	800	109	~235	0·69	—
				~275	0·38	—
				$\lambda_{max.}$ 285	0·40	SC
				~320	0·56	—
				$\lambda_{max.}$ 327	0·59	—
8	E	200	0·4	~230	54	—
				$\lambda_{max.}$ 275	27	—
				~285	25	—

Table 7. *Chromatography after acetylation of the main sterol fraction from sheep-kidney unsaponifiable matter (fourth batch)*

Wt. used, 156 mg. Alumina column, 7·6 cm. × 1·2 cm. Alumina, 10 g. (Brockmann 3).

Fraction	Eluent	Vol. (ml.)	Wt. of solute (mg.)	Absorption spectrum (*cyclo*hexane) (mμ)	$E^{1\%}_{1\,cm.}$	Remarks
1	1% E/P	250	153·7	~320	0·35	—
				$\lambda_{min.}$ 327	0·37	—
2	4% E/P	300	0·9	~230	43·5	—
				$\lambda_{max.}$ 275	21·1	SC
				~282	19·6	—
				~315	8·3	—
3	8% E/P	200	0·2 (?)	$\lambda_{max.}$ 232	103	—
				$\lambda_{max.}$ 275	52	SC
				~282	50	—
				~320	23·5	—
4	E	200	0·4	~230	78	—
				~270	42	—
				~285	35	—

~ = inflexion.

Table 8. *Chromatography of cat-kidney unsaponifiable matter*

Wt. used, 76·4 mg. Alumina column, 7·7 cm. × 1·3 cm. Alumina, 10 g. (Brockmann 3).

(*a*)

Fraction	Eluent	Vol. (ml.)	Wt. of solute (mg.)	Absorption spectrum (*cyclo*hexane) (mμ.)	$E^{1\%}_{1\,cm.}$
1	P	100	3·1	$\lambda_{max.}$ 227	30·0
				260	8·66
				350	1·38
				370	1·45
				390	1·06
2	2% E/P	100	2·4	~240–290	5·05
3	4% E/P	100	0·2	$\lambda_{max.}$ 272	168
				~320	26·5
4	6% E/P	100	1·8	$\lambda_{max.}$ 292	16·95
				~295	16·6
5	8% E/P	50	1·9	$\lambda_{max.}$ 292	4·32
6	20% E/P	300	45·7	~230	33·3
				~320	90·7
				$\lambda_{max.}$ 327	95·2
7	E	100	0·9	~230	31·9
				~245	23·8
				$\lambda_{max.}$ 280	19·05

(*b*) Rechromatography of fraction 6 after acetylation. Alumina column, 7·8 cm. × 0·9 cm. (5 g.).

1	1% E/P	175	47·3	~260	1·90
				~320	6·53
				$\lambda_{max.}$ 327	6·78
2	4% E/P	150	0·8	~230	32·8
				$\lambda_{max.}$ 277	22·5 SC
				285	22·5
				~310–320	14·1
3	8% E/P	150	0·5	$\lambda_{max.}$ 232	59·0
				~279	54·8
				$\lambda_{max.}$ 285	55·7
4	E	100	1·0	~230	20·9
				$\lambda_{max.}$ 260	17·55
				~270	17·50

~ = inflexion.

turned out to be a tocopherol (probably α), was also detected. Table 6 illustrates the chromatographic separation.

It will be seen that following SA there is a fraction (5) with $\lambda_{max.}$ 290 mμ due to tocopherol. Table 7 shows how the presence of SC was verified by rechromatography of the acetylated sterol fraction from the fourth batch of sheep kidney.

Table 8 illustrates the chromatographic separation conducted on unsaponifiable matter from cat kidney. Fraction 3 is a typical SA fraction and the principal absorption peak in fractions 4 and 5 occurs at 292 mμ. Fraction 6 shows that although sterol is the major constituent in respect of weight the absorption spectrum is very clearly that of vitamin A. The acetylated sterol fraction was rechromatographed and the vitamin A acetate absorption reappears in the least polar fraction as expected. SC was perhaps present in fraction (*b*)2 which contained an interfering vitamin A derivative and the absorption of fractions (*b*)3 and (*b*)4 indicates the presence of other substances in small amounts.

As cat kidney was the best source of the substance showing $\lambda_{max.}$ 292 mμ, $\lambda_{min.}$ 255–260 mμ (in *cyclo*hexane), it was decided to use this tissue in order to establish the nature of the compound. Table 9 indicates how one batch was concentrated.

Two lots of cat kidney weighing 460 and 1221 g. respectively were worked up. The unsaponifiable fractions were prepared and chromatographed to concentrate the SE fraction ($\lambda_{max.}$ approx. 290 mμ). The best fractions were combined and partitioned between light petroleum and 85% ethanol (15 vol. of water, 85 vol. of ethanol). The best fraction obtained showed $\lambda_{max.}$ 297 mμ, $E^{1\%}_{1\,cm.}$ 51·5, persistence ($E_{max.}/E_{min.}$) 4·2. The concentrate from the second batch was acetylated and rechromatographed, giving one fraction exhibiting three maxima at 278, 284 and 287 mμ with $E^{1\%}_{1\,cm.}$ 10·55, 11·86, 11·64 respectively. The spectrum corresponded exactly with that of α-tocopheryl acetate diluted with optically transparent material.

α-Tocopherol was obtained by saponifying α-tocopheryl acetate (synthetic) in the presence of pyrogallol. It showed in *cyclo*hexane $\lambda_{max.}$ 297 mμ, $\lambda_{max.}$ 291–292 mμ and $\lambda_{min.}$ 257–258 mμ ($E^{1\%}_{1\,cm.}$ at 297 mμ, 90·8). The best fraction from cat kidney showed exactly the same spectrum except that the intensity of absorption ($E^{1\%}_{1\,cm.}$ 51·5 at 297 mμ) was lower, indicating the presence of a transparent congener. α-Tocopheryl acetate in *cyclo*hexane shows peaks at 279 and 285 mμ with a sharp break at 287 mμ and a minimum at 255 mμ. This curve is exactly reproduced by the acetate of the product from cat kidney. α-Tocopherol was oxidized by means of ethanolic ferric chloride (John, Dietzel & Emte, 1939) to give α-tocopherylquinone ($\lambda_{max.}$ 261 mμ and 270 mμ, $E^{1\%}_{1\,cm.}$ 396 and 391 respectively). The best fraction from cat kidney gave the same two bands with $E^{1\%}_{1\,cm.}$ values 254 and 248. The effect of alkali on tocopherol is first to increase the absorption near 320 mμ and to reduce that near 290 mμ. There is room for further study of this effect, but the cat kidney product and tocopherol do not behave differently.

There can be no doubt from the foregoing that cat kidney contains vitamin E in relatively large amount.

Distribution of vitamins A and E in cat kidney. Four batches of cat kidneys were obtained from the Department of Physiology. Individual kidneys were dissected so as to separate medulla and cortex as cleanly as possible. In four experiments the medulla accounted on the average for 22% of the weight and the cortex for 78%. In another test the whole kidney was found to contain 4·3% of lipid soluble in hot ethanol, but only 10·5% of this lipid occurred in the medulla.

Table 9. *Chromatography on a larger scale of cat-kidney unsaponifiable matter*

Wt. of kidneys, 460 g. Wt. of unsaponifiable matter used, 1·5 g. Alumina column, 10·5 cm. × 3·5 cm. Alumina, 100 g. (Brockmann 3). If $E^{1\%}_{1\,cm.}$ (290 mμ) for α-tocopherol is taken as 90 the amount of vitamin E in fractions 4 (*a*), 4 (*b*) and 5 is approximately 14 mg. or 3 mg./100 g.

Fraction	Eluent	Vol. (ml.)	Wt. of solute (mg.)	Absorption spectrum (*cyclo*hexane) (mμ)	$E^{1\%}_{1\,cm.}$
1	P	500	—	—	—
2	2% E/P	500	solutes discarded	—	—
3	4% E/P	800			
4 (*a*)	6% E/P	300	51·6	$\lambda_{max.}$ 287	14·3
				297	13·4
4 (*b*)	6% E/P	200	22·1	$\lambda_{max.}$ 290	15·0
				297	15·0
5	8% E/P	700	68·7	$\lambda_{max.}$ 287	3·4
				~295	3·0

Table 10. *Vitamin A in medulla and cortex of cat kidney*

Expt.	Tissue	Vitamin A	
		Tissue (i.u./g.)	Whole kidney (i.u./g.)
1	Cortex	140	117
	Medulla	36·3	—
2	Cortex	47·7	39·3
	Medulla	8·2	—
3	Cortex	32·6	24·6
	Medulla	Not found	—
4	Cortex	137	117
	Medulla	31·9	—

Table 10 shows that vitamin A is preferentially located in the cortex to an extent greater than would be expected from the distribution of lipid. Expts. 2 and 3 show vitamin A values lower than in the earlier work but still high compared with other species. Vitamin E was not found here in the kidney cortex. The unsaponifiable materials from Expt. 1 were chromatographed and it appeared that fraction 4 (eluted by 6% ether–light petroleum) contained both SA and vitamin E. The solute was dissolved in a little methanol and left at $-20°$ for 1 hr. The solution was filtered and the methanol-insoluble material was found to contain SA ($\lambda_{max.}$ 272 mμ) and the methanol-soluble fraction to contain vitamin E ($\lambda_{max.}$ 290 mμ, inflexion at 297 mμ).

The estimated vitamin E content of the whole kidney was 1·05 mg./100 g.; this was all in the cortex. Similarly for Expt. 4 the whole kidney contained 1·31 mg./100 g. and the cortex 1·62 mg./100 g., with negative results for the medulla. Although the two vitamins tend to occur together there are obvious fluctuations from one individual to another in the amounts of vitamins A and E in cat kidney. The absence of vitamin E from the medulla is, however, a striking result worthy of further investigation.

DISCUSSION

Kidney tissue for most species contains only very small amounts of vitamin A. This circumstance makes it easier to achieve a sharp fractionation of kidney unsaponifiable than of liver unsaponifiable matter.

Both SA and SC are present in the kidneys of most of the species studied. The units used in Table 4 are such that for vitamin A, 3 i.u. = 1 μg.; for SA, 1 unit = 6 μg.; and for SC, 1 unit = 11 μg. These figures are based upon the provisional assumption that our best preparations of SA and SC are not far from pure. Thus pig kidney contains (per gram) about 1·6 μg. of vitamin A, 16·8 μg. of SA and 8·8 μg. of SC.

In cat kidney the vitamin A content on the average is about 33 and the vitamin E content 25 μg./g. Although there must obviously be variations from one individual to another, the figures obtained are high, and the evidence that no vitamin E can be found in the medulla even when there is a good deal in the cortex suggests that the peculiarities of cat kidney are worthy of much more study.

The broad picture presented here carries the implication that similar work on diseased kidneys will be fruitful. For this it is perhaps best to work on human tissue.

SUMMARY

1. It has been shown that SA and SC (two spectroscopically recognizable compounds of unknown composition) are normal constituents of the unsaponifiable fraction of kidneys from eleven different species; the amounts detected in all cases were of the order 20 μg./g. for SA and 5 μg./g. for SC.

2. In general it was found that kidney tissue contains only a few i.u. of vitamin A/g., but the results with cats were quite unusual. It was shown that cat kidneys contain an average of approximately 100 i.u. of the vitamin/g.

3. Vitamin E was detected in cat, dog and sheep kidney, the concentration in cat kidney being much greater than in the other two species.

4. A study of the distribution of vitamins A and E in cat kidney revealed that they were to be found predominantly in the cortex. Using the techniques described in the text, vitamin E was not detected in the medulla.

One of us (J.S.L.) participated in the work as holder of an I.C.I. Fellowship.

We are indebted to the Nuffield Foundation for financial assistance towards the cost of the investigation.

REFERENCES

Heaton, F. W., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **60**, xviii.

Heaton, F. W., Lowe, J. S. & Morton, R. A. (1957). *Biochem. J.* **67**, 208.

John, W., Dietzel, E. & Emte, W. (1939). *Hoppe-Seyl. Z.* **257**, 173.

Mervyn, L. (1956). Ph.D. Thesis: University of Liverpool.

Morton, R. A. & Stubbs, A. L. (1942). *Analyst*, **71**, 348.

Cholesterol and Liver Vitamin A in Male and Female Rats

By B. GREEN, A. A. HORNER, J. S. LOWE and R. A. MORTON
Department of Biochemistry, The University of Liverpool

(*Received* 8 *January* 1957)

Cholesterol feeding has in recent years been widely used in the experimental approach to a variety of problems, including atherosclerosis. The rat shows a remarkable ability to remove absorbed cholesterol from the plasma and store it in the liver. If cholesterol is fed over a period, much of it, in the rat, is stored in the liver. This animal has only a limited ability to convert exogenous cholesterol into cholic acid and so to remove it from the liver via the bile. Cholesterol esters therefore accumulate, together with other lipid matter, producing the well-known 'cholesterol fatty liver' (Chanutin & Ludewig, 1933).

Okey, Gillum & Yokela (1934) reported that male rats stored significantly more cholesterol in the liver than females fed cholesterol at the same dietary level.

The present paper records another aspect of cholesterol metabolism in the rat in which the sexes differ markedly. It was shown earlier, incidentally to other work (Kantiengar & Morton, 1955), that adult male rats fed on a diet containing 2% cholesterol for a period of 8 weeks had considerably lower liver vitamin A stores than control male rats receiving no cholesterol. In that work, both experimental groups consisted of five rats; the livers of each group were bulked for estimation of vitamin A. Further experiments, using more animals, have been performed, and the results have been analysed statistically. The first two experiments of this type (Green, 1955), using weanling and adult female rats, did not agree with the earlier findings, but the possibility remained that the disagreement was due to a sex difference. Four further experiments have therefore been performed, using weanling and adult rats of both sexes. These six experiments establish a clear-cut difference in the effect of cholesterol feeding on liver vitamin A storage in male and female rats.

EXPERIMENTAL

Animals and diets. Newly weaned or adult hooded rats were given either a synthetic diet (*A*) or one based on a compound cube ration (Animal diet no. 4, British Extracting Co. Ltd., Bromborough, Cheshire). The basal diets were made up as follows. Diet *A*: casein (Genatosan Ltd., 'low vitamin content') 18, maize starch 65, arachis oil 5, Marmite water-soluble yeast extract 8, salt mixture (Osborne & Mendel, 1913) 4%. Diet *B*: food cubes were ground to powder, exhaustively extracted with diethyl ether, and to the dried product was added arachis oil (10%) containing α-tocopherol (3 mg. per 100 g. of diet). Diet *C*: food cubes were ground (but not extracted) and arachis oil (5%) was incorporated.

Procedure. Experiments were carried out with separate groups of newly weaned and adult rats of both sexes. In each experiment there were two experimental groups. Group I received the basal diet, whilst group II received the same diet plus 2% of crystalline cholesterol (Croda Ltd.). This was dissolved in an amount of arachis oil equivalent to 5% of the diet. The groups were of equal size, as nearly as was practicable with available animals, and each rat in group I had a corresponding litter-mate in group II. The individual experiments were planned as follows. (They are described in chronological order.) (i) Six females in each group were fed on diet *A*, from weaning, for 5–6 weeks. (ii) Ten adult females in each group were fed on diet *B* for 6 weeks. (iii) Female rats, eight in group I and six in group II, were fed on diet *C*, from weaning, for 8–9 weeks. (iv) Male rats, four in group I and five in group II, were fed on diet *C*, from weaning, for 8–9 weeks. (v) Fourteen adult males in each group were fed on diet *C* for 8–9 weeks. (vi) Twelve adult females in each group were fed on diet *C* for 8–9 weeks.

The rats were housed separately in wire-mesh cages with raised screen floors to prevent coprophagy. They were fed a weighed amount of food in individual pots. This was initially 8 g./day for weanling rats, increasing to 10 g. after 3 weeks. The adult ration was always 12 g./day. Water was freely provided.

Vitamin A dosage. Diets *A* and *B* were vitamin A-free. In Expts. (i) and (ii) all animals were therefore given a tested cod liver oil, suitably diluted in arachis oil and administered orally by pipette (0·2 ml.). In Expt. (i) the dose provided 100 i.u. of vitamin A daily. In Expt. (ii) the same dose was administered once every 5 days.

The unextracted food cubes used in diet *C* contained approximately 5 i.u. of vitamin A/g. As the animals consistently ate all their food, it was unnecessary to give vitamin A by mouth in the four experiments in which diet *C* was fed.

Analysis of livers

At the end of the feeding period the rats were killed by chloroform anaesthesia and the livers were removed, washed, dried on filter paper, and weighed.

Saponification. Each liver was separately saponified by the following method. To the moist tissue, cut into small pieces, 60% (w/v) KOH (0·5 ml./g.) was added and digestion continued on a boiling-water bath until the mixture was homogeneous. Ethanol (95%, 2 vol.) was then added, and the mixture boiled under reflux for a further 45 min. Water (4 vol.) was then added and the mixture extracted (five or

six times) with ether (freshly distilled over reduced Fe). The combined ether extracts were washed with water until free from alkali, dried over anhydrous Na_2SO_4, filtered under suction through a sintered-glass funnel, taken to dryness under N_2, and weighed.

Vitamin A analyses. The liver unsaponifiable material was dissolved in spectroscopically pure *cyclo*hexane, and the liver vitamin A content determined by the method of Cama, Collins & Morton (1951), with a Unicam SP. 500 spectrophotometer.

Liver cholesterol analyses. In Expts. (i) and (ii) liver cholesterol content was determined on each liver unsaponifiable fraction by the method of Kenny (1952) as modified by Green, Lowe & Morton (1955).

RESULTS

In all six experiments, livers from group II animals were markedly larger than those of the corresponding group I animals. Group I livers were normal in appearance, but group II animals invariably had typical pale pink-grey 'cholesterol fatty livers'. These contained greatly increased amounts of unsaponifiable material. There was a similar increase in cholesterol content (Expts. (i) and (ii)). These findings are shown in Tables 1 and 2.

A direct comparison can be made between the increases in liver unsaponifiable material of young males and females of group II in Expts. (iv) and (iii) respectively, as these animals received the same diet for the same length of time. Both sexes showed the same increase in group II total unsaponifiable matter when compared with their group I controls. The increase was 10·0 times for the males and 9·7 times for the females.

A similar comparison is possible between the adult males and females of Expts. (v) and (vi). In the case of adults a sex difference was observed. The increase in total liver unsaponifiable material of group II over group I animals in these cases was 6·5 times for the males and 9·5 times for the females.

The mean liver vitamin A contents for each group and the statistical analyses derived from the individual values for each animal within a group are shown in Tables 3 and 4.

DISCUSSION

The data given in Tables 3 and 4 show first that feeding cholesterol does not affect liver vitamin A storage in adult female rats, but lowers the liver vitamin A levels of adult males. In earlier work in this Laboratory (Kantiengar & Morton, 1955) the cholesterol-fed adult males had 35 % less vitamin A in bulked livers than had the controls. In the present work (Expt. (v), Table 4) single livers were examined and the average drop was less dramatic (16 %), but highly significant even at the 1 % level of probability.

Secondly, the sex difference observed with adults also applies to young rats fed on cholesterol from weaning. Here the results are less clear-cut. In Expts. (i) and (iii) (Table 3) the cholesterol-fed

Table 1. *Liver weights, liver unsaponifiable material, and liver cholesterol contents of control (group I) and cholesterol-fed (group II) young male and female rats*

	Males Expt. (iv)		Females Expt. (i)		Females Expt. (iii)	
	Group I	Group II	Group I	Group II	Group I	Group II
No. of rats	4	5	6	5*	8	6
Mean liver wt. (g.)	6·14	7·29	4·85	6·48	5·93	6·31
Percentage of unsaponifiable matter	0·55	4·65	0·96	10·75	0·43	4·06
Total unsaponifiable matter (mg.)	33·8	339	47	695	26	253
Total cholesterol (mg.)	—	—	16·4	483	—	—

* One animal in group II was incorrectly sexed at the time of weaning; therefore its analyses have been omitted from the mean values.
— signifies not determined.

Table 2. *Liver weights, liver unsaponifiable material, and liver cholesterol contents of control (group I) and cholesterol-fed (group II) adult male and female rats*

	Males Expt. (v)		Females Expt. (ii)		Females Expt. (vi)	
	Group I	Group II	Group I	Group II	Group I	Group II
No. of rats	14	14	10	10	12	12
Mean liver wt. (g.)	8·18	9·60	6·83	7·28	7·49	8·43
Percentage unsaponifiable matter	0·50	2·77	0·99	3·33	0·56	4·74
Total unsaponifiable matter (mg.)	41	266	68	243	42	401
Total cholesterol (mg.)	—	—	25	206	—	—

Table 3. *Comparison of liver vitamin A levels in control (group I) and cholesterol-fed (group II) young male and female rats*

Expt.	Sex	No. of rats I	No. of rats II	Mean liver vitamin A (i.u.) I	Mean liver vitamin A (i.u.) II	Percentage difference $\left(\frac{I-II}{I}\times 100\right)$	Statistics t (found)	Statistics t (tables)	
(iv)	M.	4	5	1699 (±87)*	924 (±58)	46	8·171	3·499	S.
(i)	F.	6	5	796 (±73)	646 (±93)	19	0·953	3·250	N.S.
(iii)	F.	8	6	1249 (±119)	952 (±78)	24	1·930	3·055	N.S.

* Standard deviation from the mean. S. = significant. N.S. = not significant. For significance, t (found) must exceed t (tables) at the desired level of probability (0·01) (Snedecor, 1946).

Table 4. *Comparison of liver vitamin A levels in control (group I) and cholesterol-fed (group II) adult male and female rats*

Expt.	Sex	No. of rats I	No. of rats II	Mean liver vitamin A (i.u.) I	Mean liver vitamin A (i.u.) II	Percentage difference $\left(\frac{I-II}{I}\times 100\right)$	Statistics t (found)	Statistics t (tables)	
(v)	M.	14	14	3714 (±494)*	3117 (±524)	16	3·100	2·779	S.
(ii)	F.	10	10	5438 (±740)	5806 (±890)	−7	0·437	2·878	N.S.
(vi)	F.	12	12	5003 (±484)	5300 (±854)	−6	0·651	2·819	N.S.

* See footnote to Table 3.

young females have liver vitamin A stores lowered, on the average, by 19 and 24 % respectively, but these changes are not significant at $P = 0{\cdot}01$. The mean depression in Expt. (i) is not significant even at the 10 % level of probability. The mean depression in Expt. (iii) is just significant at the 10 % level [t (found) = 1·93, t (tables) = 1·78], but not at the 5 % level [t (tables) = 2·18]. With young males, however, a dramatic drop of 46 % is highly significant at the 1 % level of probability.

If the possibility that dietary cholesterol affects intestinal absorption of vitamin A is excluded, two alternative reasons can be advanced why cholesterol-fed rats should store less vitamin A in their livers than do controls: (1) cholesterol-fed rats require more of the vitamin, i.e. they metabolize it more rapidly; (2) absorbed cholesterol competes with vitamin A for storage in the liver, and may even displace it.

If the production of a 'cholesterol fatty liver' is itself a metabolic hazard, the first suggestion can be supported. Thus the stress of sterile abscesses (induced by subcutaneous injection of turpentine) caused a marked lowering of serum and liver vitamin A level (Kagan & Kaiser, 1955). Ershoff (1950) showed that the stress of sustained exposure to cold accelerated the production of vitamin A deficiency. Clark & Colburn (1955) demonstrated that the stress of inanition mobilized liver vitamin A and suggested that the effect was mediated through the adrenal glands.

There is indirect support for the second idea, namely that absorbed cholesterol competes with vitamin A for liver storage 'space'. Cholesterol and vitamin A ester both pass from the plasma to the parenchymal cells via the reticulo-endothelial system. For example, Friedman & Byers (1954) blocked the reticulo-endothelial system with colloidal indian ink and then administered cholesterol, which accumulated in the plasma. Glover & Morton (1948) showed that chylomicrons containing vitamin A are ingested from the blood by the Kupffer cells. Several workers (e.g. Lasch & Roller, 1936) have reported that reticulo-endothelial system blocking agents displace vitamin A into the plasma. Therefore excessive ingestion of cholesterol-bearing chylomicrons by the Kupffer cells may cause ejection of vitamin A from these cells.

When the animals in Expt. (i) were killed, the kidneys, lungs and spleen were examined to see

whether vitamin A had been displaced from the liver to other organs. No such effect was observed. As females were used in that experiment, it is now necessary, in view of our later findings, to examine the same organs from cholesterol-fed males.

Although Okey *et al.* (1934) reported a difference in the amounts of exogenous cholesterol stored in the liver by males and females, these amounts were large in both sexes. Therefore the depression of liver vitamin A in only males cannot readily be explained in terms of a direct displacement by cholesterol. Cholesterol feeding must have some indirect metabolic effect on the liver vitamin A levels of male rats.

A well-known sex-difference in the rat is the much higher requirement of males for the essential fatty acids (see e.g. Greenberg, Calbert, Savage & Deuel, 1950). One of these acids (linoleic) appears to be the main acid present in plasma cholesteryl esters (Deuel, Alfin-Slater, Wells, Kryder & Aftergood, 1955), and Peifer & Holman (1955) showed that a dietary cholesterol supplement accelerated the rate of induction of essential fatty acid deficiency. The sex difference in liver vitamin A storage may therefore be no less closely linked with the essential fatty acids than with cholesterol.

Sexually immature males fed on cholesterol showed a greater depression of liver vitamin A than adult males. Coleman, Chen, Alfin-Slater & Deuel (1956) castrated mature male rats and then fed them on a diet containing cholesterol (1%) and cottonseed oil (15%). After 6 weeks the castrated males had less liver cholesterol than intact male controls. However, ovariectomized females showed the reverse effect. If rats of either sex were castrated at weaning, and subsequently, at an age when their intact controls were sexually mature, given the same diet, no significant differences in liver cholesterol levels were observed for either sex, when compared with intact controls.

The reason for the sex difference in essential fatty acid requirement is not yet known. Panos, Klein, Wall & Finerty (1956) showed that the retardation of growth which is typical of fat deficiency is less marked in ovariectomized females, or intact females given testosterone, than in intact females, but is more marked in ovariectomized females given oestrone. Similar investigations with males are necessary before an interpretation of these results or of those of Coleman *et al.* (1956) can be profitably attempted. The recent work emphasizes the interrelationship of cholesterol, the essential fatty acids and gonadal function in both sexes.

We are now carrying out experiments to determine whether there is a sex difference in liver vitamin A storage in fat-deficient rats, and in fat-deficient rats fed cholesterol.

SUMMARY

1. The effect of a dietary supplement of cholesterol (2% by weight) on liver vitamin A storage has been determined for young and adult rats of both sexes.

2. Cholesterol feeding had no statistically significant effect on liver vitamin A stores in young or adult female rats, but the liver vitamin A levels of males were significantly lowered.

3. The mean depression of liver vitamin A was more marked in sexually immature males (46%) than in adult males (16%).

Acknowledgement is made to the Nuffield Foundation for a grant which allowed one of us (A.A.H.) to participate in this work, to the Medical Research Council for a Scholarship (B.G.) and to Imperial Chemical Industries Ltd., for a Fellowship (J.S.L.).

REFERENCES

Cama, H. R., Collins, F. D. & Morton, R. A. (1951). *Biochem. J.* **50**, 48.
Chanutin, A. & Ludewig, S. (1933). *J. biol. Chem.* **107**, 207.
Clark, I. & Colburn, R. W. (1955). *Endocrinology*, **56**, 232.
Coleman, R. D., Chen, Y.-M., Alfin-Slater, R. B. & Deuel, H. J. jun. (1956). *Fed. Proc.* **15**, 234.
Deuel, J. H. jun., Alfin-Slater, R. B., Wells, A. F., Kryder, G. D. & Aftergood, L. (1955). *J. Nutr.* **55**, 337.
Ershoff, B. H. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 586.
Friedman, M. & Byers, S. O. (1954). *Amer. J. Physiol.* **179**, 201.
Glover, J. & Morton, R. A. (1948). *Biochem. J.* **43**, xii.
Green, B. (1955). Ph.D. Thesis: University of Liverpool.
Green, B., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **61**, 447.
Greenberg, S. M., Calbert, C. E., Savage, E. E. & Deuel, H. J. jun. (1950). *J. Nutr.* **41**, 473.
Kagan, B. M. & Kaiser, E. (1955). *J. Nutr.* **57**, 277.
Kantiengar, N. L. & Morton, R. A. (1955). *Biochem. J.* **60**, 28.
Kenny, A. P. (1952). *Biochem. J.* **52**, 611.
Lasch, F. & Roller, D. (1936). *Klin. Wschr.* **15**, 1636.
Okey, R., Gillum, H. L. & Yokela, E. (1934). *J. biol. Chem.* **107**, 207.
Osborne, T. B. & Mendel, L. B. (1913). *J. biol. Chem.* **15**, 317.
Panos, T. C., Klein, G., Wall, R. L. & Finerty, J. C. (1956). *Fed. Proc.* **15**, 141.
Peifer, J. J. & Holman, R. T. (1955). *Arch. Biochem. Biophys.* **57**, 520.
Snedecor, G. W. (1946). *Statistical Methods*, chap. 3, p. 62. Ames, Iowa: Iowa State College Press.

Enzymic Oxidation of Catechin to a Polymer Structurally Related to some Phlobatannins

By D. E. HATHWAY and J. W. T. SEAKINS
The British Leather Manufacturers' Research Association, Milton Park, Egham, Surrey

(*Received* 15 *January* 1957)

The formation of polymers and hydrogen peroxide during the autoxidation at 35° and pH 6–8 of catechin and of related 3′:4′-dihydroxyflavans has been studied by measurement of oxygen uptake, and by the elementary analyses, absorption spectra and reactions of the dialysed polymers (Hathway & Seakins, 1957*a*). The evidence obtained, together with that provided by the spectroscopic study of intermediates produced by silver oxide oxidation (Hathway & Seakins, 1955), supports the quinone polymerization mechanism for catechin autoxidation. Whereas oxidative coupling of 5:7-di-*O*-methylcatechin resembles that of catechol, quinone polymerization of catechin and of 5:7:3′:4′-tetrahydroxyflavan involves the phloroglucinol residue.

The mechanism for the autoxidation of catechin leading to a recognizable polymer has now been reported (Hathway & Seakins, 1957*a*), but as there was little information relating to the enzymic oxidation of catechin and no information concerning the product (Roberts & Wood, 1950), the aerobic oxidation of catechin to a polymer precisely similar to the autoxidation polymer has now been investigated by using different plant polyphenoloxidases. Since *Uncaria gambir* Roxb. (Rubiaceae) leaves and *Acacia catechu* Willd. (Leguminosae) heartwood are known to contain large quantities of catechin and its epimeride respectively, the tannin extractives of these plant tissues have been examined for the occurrence of phlobatannins related to the polymers from the autoxidation and polyphenoloxidase oxidation of catechin. It should, however, be stated that as these phlobatannins were isolated from harvested plant materials, they may not be localized within living tissues of the plants, but may have arisen subsequently by enzymic or chemical change or both. This possibility does not affect the work to be described, which is concerned with the biogenesis and structure of the phlobatannins produced under the conditions encountered. In general, plant phenolics, including phlobatannins or their precursors which originate in the cambium, migrate into the dead heartwood and bark tissues (Erdtman, 1956; Hillis, 1956; Jaccard, 1938). Abscission stimulates phlobatannin formation in a seasonal crop such as leaves (Forsyth, 1952*b*; Goris, 1907; Roberts, 1952). The plants with which this work is concerned are valued for their phlobatannin extractives.

EXPERIMENTAL

General. Evaporations were carried out in N_2 under reduced pressure at <35°. Paper chromatography was carried out at 23±2° in all-glass apparatus. Chromatograms were dried at room temperature, unless otherwise stated. A Hanovia mercury-arc lamp fitted with a Wood's-glass filter was used to examine chromatograms for fluorescent zones. Hide-powder tests were made by the method of Grassmann, Endisch & Kuntara (1951).

Acetone-dried powders of Psalliota campestris *and* Nicotiana tabacum. Freshly picked mushrooms (350 g.) were plunged into ice-cold acetone (2 l.), sliced, and homogenized in a top-drive macerator. The homogenate obtained was filtered through sintered glass and the solid (20 g.) was immediately washed five times with 500 ml. portions of ice-cold acetone, and dried at 0° *in vacuo*. This powder (2·8 enzyme units) had a polyphenoloxidase activity of purpurogallin number 0·14. The purpurogallin number (P.N.) was determined by the method of Keilin & Mann (1938) but purpurogallin was estimated in ethanol solution at 375 and 430 mμ by means of a standard u.v.-light spectrophotometer. One enzyme unit (E.U.) corresponds to the quantity of enzyme which produces 1 g. of purpurogallin in 5 min. at 20°.

The acetone-dried powder of *Psalliota campestris* was used without further treatment for enzyme-catalysed oxidations, whereas the acetone-dried powder of *Nicotiana tabacum*, P.N. 0·0024, was first eluted with ice–water (10 ml./250 mg.) until free from amino acids.

Potato juice. A chilled potato was minced and pulverized, and the juice expressed was filtered, chilled, and centrifuged to remove starch (Raper & Wormall, 1923). Freshly prepared juice had polyphenoloxidase activity of P.N. 0·0022, and was used immediately or after preliminary dialysis in Visking 18/32 seamless cellulose tubing.

Manometry. Use was made of Haldane's (1921) constant-pressure respirometer, in which the reaction vessel was connected by small-bore pressure tubing to a gas-burette and to a constant-pressure manometer, the other limb of which was connected to a compensation vessel. Both the reaction and the compensation vessels were shaken at 20° at a speed sufficient to maintain the reaction mixture as a foam. *n*-Butyl phthalate was used in the constant-pressure manometer and gas-burette.

Isolation of tannins from Acacia catechu *and* Uncaria gambir. A 5% (w/v) aqueous solution (400 ml.) of extract (containing 43% of the total solids as tannin, by hide-powder test) prepared from *Acacia catechu* heartwood was

extracted for 2 days with ether and then for 5 days with ethyl acetate. Progress of solvent extraction was followed by means of two-dimensional paper chromatography. For this purpose, samples (5 μl.) of the test solutions were spotted at a distance of 2 cm. from both edges of the lower left-hand corner of Whatman no. 1 filter papers, 25·5 cm. square, and chromatographed by the ascending method with N-acetic acid as first-way solvent and *n*-butanol–acetic acid–water (6:1:2, by vol.) as second-way solvent.

Extraction with ether was continued until catechin, R_F 0·54, 0·74, and *epi*catechin, R_F 0·49, 0·62, had been removed. The ether residue (6 g.) constituted 30% (by wt.) of the whole extract. In addition to catechin epimers this fraction contained flavonols and substances of unknown composition. Extraction with ethyl acetate was similarly continued until other substances of unknown composition had been removed. The ethyl acetate residue (4·6 g.) constituted 23% (by wt.) of the original extract. The residual aqueous solution contained 58% of the total tannin (determined by hide-powder test), and paper chromatography showed a single phenolic component at the origin. This tannin was precipitated from dilute solution either by 1% (w/v) gelatin reagent, or when refluxed with a mixture of formalin (40% formaldehyde) and 12N-HCl. In order to recover an analytical sample of this tannin from the residual aqueous solution, deionization was first carried out by electrodialysis. The aqueous solution was accordingly transferred to the middle compartment of a cell, constructed from Pyrex pipe-line components (Lovering & Smith, 1946), but fitted with a Permaplex A-10 anion-exchange membrane (The Permutit Co. Ltd., London, W. 4) and a Permaplex C-10 cation-exchange membrane. The apparatus was supplied with 100v d.c. until after 30 hr. the resistance approached a limiting value. Precipitation of tannin paralleled deionization. The supernatant was centrifuged off, and the sediment was washed with five successive 50 ml. vol. of water, and centrifuged after each addition and the supernatants were discarded. The phlobatannin, which was dried over P_2O_5 at 20° *in vacuo* for 3 days and at 70° *in vacuo* for 8 hr., did not give the ferric and vanillin reactions.

A 5% (w/v) aqueous solution (400 ml.) of extract (containing 40% of the total solids as tannin, by hide-powder test), derived from *Uncaria gambir* leaves by water percolation, was similarly extracted with solvents. The ether residue (9 g.) and the ethyl acetate residue (2·5 g.) constituted 45 and 12·5% (by wt.) respectively of the original extract. Catechin (8·6 g.) crystallized from a solution of the ether residue in aqueous acetone, forming needles, m.p. 176–177°, undepressed by admixture with an authentic specimen. The residual aqueous solution accounted for 65% of the total tannin (hide-powder test), and paper chromatography revealed a single phenolic component at the origin. This tannin was precipitated from dilute solution either by 1% (w/v) gelatin reagent, or by refluxing with a mixture of formalin (40% formaldehyde) and 12N-HCl. The phlobatannin which was recovered after deionization and elution as described above, was dried at 70° *in vacuo*, and did not give the ferric and vanillin reactions.

5-Methoxy-4-methylresorcinol. A specimen, m.p. 120° (Found: C, 62·1; H, 6·7. Calc. for $C_8H_{10}O_3$: C, 62·4; H, 6·5%), was prepared by the method of Robertson & Whalley (1951).

Separation of the flavonols from Acacia catechu *by paper chromatography.* Spots (10 μl.) of a 2% (w/v) solution of the ether residue in ethanol were applied to base lines, 4 cm. from the lower edge of five sheets of Whatman no. 3 filter paper 57 cm. long. Single-way ascending chromatography was effected with N-acetic acid. After the papers had been irrigated for 4 hr. they were dried and given a second development with N-acetic acid to ensure complete migration of the catechins. Transverse zones, containing the flavonols, were then located in u.v. light, and cut out, and extracted with boiling ethanol. Spots (5 μl.) of a 0·5% solution of the ethanol residue (3 mg.), and marker spots (5 μl.) of 0·5% solutions of fisetin, myricetin, quercetagetin, quercetin and robinetin respectively were applied to Whatman no. 1 filter papers, which were irrigated for 24 hr. by the ascending method with the following solvent systems: *m*-cresol–acetic acid–water (25:1:24, by vol.; upper phase) (Bate-Smith, 1949), and butan-2-ol–acetic acid–water (14:1:5, by vol.). Papers which had been chromatographed with the solvent system containing *m*-cresol were dried at 60°. Individual flavonols were detected by their fluorescence in u.v. light.

Isolation of quercetagetin from Tagetes erecta *flowers.* The occurrence of quercetagetin in *Tagetes erecta* flowers was observed by Mahal (1938), and the flavonol was therefore isolated from this source by the following simple procedure. Petals (40 g.) from the orange flowers were disintegrated in a top-drive macerator in 250 ml. of 95% ethanol, and the homogenate obtained was refluxed for 1 hr. The filtrate was concentrated to 20 ml. and refluxed with 100 ml. of 2N-H_2SO_4 for 2 hr., when a trace of insoluble material was removed. When residual solvent was evaporated from the aqueous phase, precipitation occurred, and the solid recovered was extracted consecutively with light petroleum (b.p. 40–60°, 800 ml.) and boiling ether (500 ml.). The petroleum extract was discarded. Dropwise addition of water to a solution of the ether residue (250 mg.) in 10 ml. of ethanol deposited traces of tar, which were removed. Further dilution of the filtrate precipitated quercetagetin, which was twice crystallized from ethanol, forming yellow needles (50 mg.), m.p. 310° (decomp.), $\lambda_{max.}$ 259 (log ϵ 4·28); 362 (log ϵ 4·31) mμ; inflexion at 272 (log ϵ 4·15) mμ (Found: C, 56·9; H, 3·4. Calc. for $C_{15}H_{10}O_8$: C, 56·6; H, 3·2%).

RESULTS

The present work consists of an exploratory study of the enzymic oxidation of catechin to a polymer, precisely similar to a polymer produced by autoxidation (Hathway & Seakins, 1957*a*) and comparable with phlobatannins which have now been isolated in high yield from amongst the tannin extractives of two plants (Hathway & Seakins, 1957*b*).

Crude enzyme preparations were used, since in many cases the polyphenoloxidase activity has been found to be identical with that for the more purified enzyme (Raper, 1932), and our early results also showed that greater difficulty in measuring the initial rate of oxidation was encountered with purified than with crude enzyme preparations.

During the aerobic oxidation of catechin by mushroom polyphenoloxidase at 20° and pH 8, the rate of O_2 uptake diminished rapidly from the time

the reaction commenced (Fig. 1). The oxidation stopped before two equivalents of O_2 had been taken up, unless a relatively large quantity of enzyme was initially present. Maximum O_2 uptake with the lowest enzyme concentration represented incomplete oxidation of catechin; and a second addition of enzyme resulted in an immediate acceleration of O_2 uptake (Fig. 1). Owing to the rapid inactivation of the enzyme during the reaction, it was difficult to obtain reliable measurements for the initial rate of oxidation, particularly if purified enzyme was used, and this experience is in agreement with the results obtained by earlier workers on the oxidation of catechol by mushroom polyphenoloxidase. To overcome this difficulty, Graubard & Nelson (1935) used the O_2 uptake at the end of 1 hr. as a measure of polyphenoloxidase activity, and the activity of the enzyme thus measured agreed with its activity similarly measured with catechol as substrate. Alternatively, by measuring O_2 uptake at 1 min.

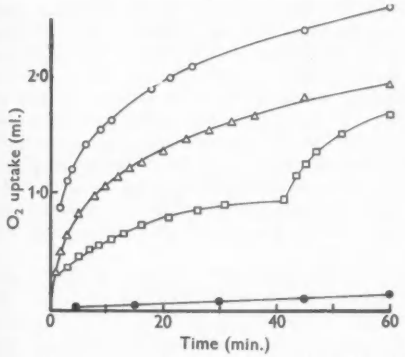


Fig. 1. Progress curves for the oxidation of catechin at 20° by mushroom polyphenoloxidase. In addition to 20 ml. of 0·07 M-phosphate buffer, pH 8, and 0·2 m-mole of catechin (●) the reaction flask contained 16 (○), 8 (△) and two separate additions of 4×10^{-3} E.U. (□). A compensatory flask contained phosphate buffer.

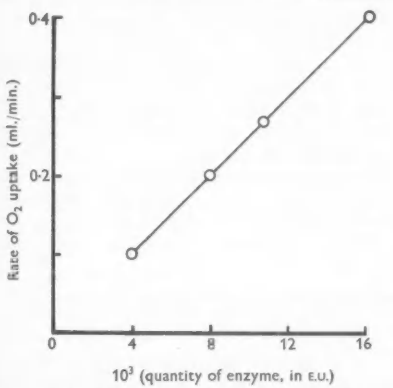


Fig. 2. Relationship between rate of O_2 uptake and enzyme concentration (mushroom enzyme).

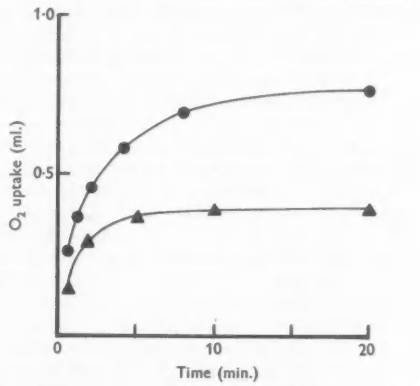


Fig. 3. Progress curves for the oxidation of catechin at 20° by potato polyphenoloxidase. Reaction flask contained 20 ml. of 0·07 M-phosphate buffer, pH 8, 0·2 m-mole of catechin and 8 (●) or 4×10^{-3} E.U. (▲). A compensatory flask contained phosphate buffer.

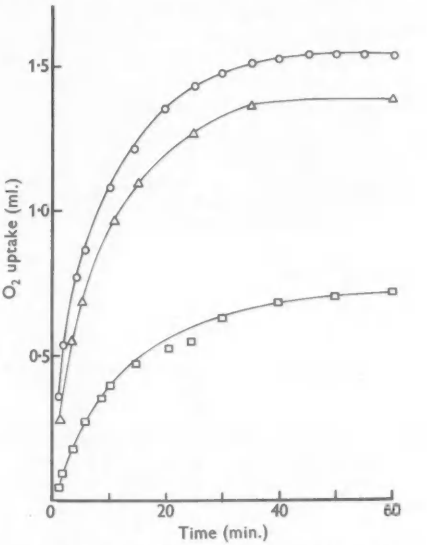


Fig. 4. Progress curves for the oxidation of catechin at 35° by tobacco polyphenoloxidase. Reaction flask contained 20 ml. of 0·07 M-phosphate buffer, pH 6, 0·2 m-mole of catechin and 2 (○), 0·7 (△) and $0·3 \times 10^{-3}$ E.U. (□). A compensatory flask contained phosphate buffer.

intervals for the first 2 or 3 min. (Keilin & Mann, 1938) and plotting the initial rate of oxidation observed against enzyme concentration, the usual linear relationship resulted (Fig. 2). The oxidation of catechin by potato polyphenoloxidase under identical temperature and pH conditions was also studied (Fig. 3), but the rate of enzyme inactivation was higher. The progress curves obtained for oxidation by tobacco polyphenoloxidase (Fig. 4) were similar to those for oxidation by mushroom enzyme, and O_2 uptake ceased before oxidation was complete unless a large amount of enzyme was present. Under the different optimum conditions of temperature (35°) and pH (6) for tobacco polyphenoloxidase (Reid, 1956), O_2 uptake at the end of 1 hr. was greater, at comparable levels of activity, for this enzyme than for the other enzymes studied.

The autoxidation and polyphenoloxidase oxidation of catechin gave polymers which had the same elementary analysis, remained at the origin in two-dimensional paper chromatography, were retained by hide-powder, and were precipitated by gelatin or on refluxing with a formalin–HCl mixture. The absorption spectra exhibited bands at 270 and 310 mμ and a shoulder at 500 mμ, but the shoulder at 500 mμ was slightly less intense with the autoxidation polymer (Fig. 5). The intensity of this shoulder decreases during the course of autoxidation, and this may be due to slight oxidative degradation by hydrogen peroxide, which is not formed during enzymic oxidation (cf. Beer, Broadhurst & Robertson, 1954). Figs. 5–7 were drawn from measurements made at 5 mμ intervals. Oxidation of catechin by hydrogen peroxide and horse-radish peroxidase gave a different polymer, which exhibited a single absorption band at 350 mμ.

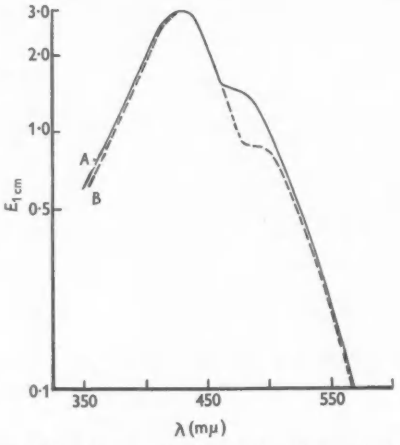


Fig. 5. Absorption spectra of catechin polyphenoloxidase oxidation (*A*) and autoxidation (*B*) polymers.

Percolation of *Acacia catechu* heartwood and freshly harvested *Uncaria gambir* leaves with water gave extracts, the residues of which contained 40% of tamin; precautions were taken to prevent autoxidation during extraction. The residual aqueous solutions were examined after exhaustive solvent extraction, and found to contain approximately 50% of the total tannin. Two-dimensional paper chromatograms in both cases revealed a single phenolic component at the origin. Electrodialysis of the residual solutions, in a cell fitted with ion-exchange membranes, precipitated the tannins, which were separated in the centrifuge after elution. Controlled drying furnished analytical specimens with elementary analyses (Table 1) similar to those for the polymer of catechin oxidation, of empirical formula $C_{15}H_{10}O_6,3H_2O$. The i.r. spectra provided evidence for hydration, for even after protracted drying at 70° *in vacuo* a strong band persisted at 1627–1635 cm.$^{-1}$. The absorption spectra of these phlobatannins at pH 8 (Fig. 6) were similar to those of the polymers of catechin oxidation, exhibiting maxima at 270, 410 and 500 mμ. These substances are retained by hide-powder, they do not give the ferric and vanillin reactions and they are precipitated by gelatin or on boiling with a formalin–HCl mixture.

The aerobic oxidation of an equimolecular mixture of *homo*catechol and phloroglucinol by mushroom polyphenoloxidase gave a polymer which showed absorption at 460 mμ (Fig. 7), in contrast to the single absorption band at 250 mμ of catechol autoxidation polymer (Hathway & Seakins 1957*a*). *homo*Catechol and 5-methoxy-4-methylresorcinol (Robertson & Whalley, 1951) substrates afforded a polymer which showed a strong absorption maximum at 480 mμ.

A flavonoid fraction derived from *Acacia catechu* heartwood was found to contain in addition to quercetin (Perkin, 1897) two flavonols, chromatographically indistinguishable in two different

Table 1. *Phlobatannin analyses*

	Found (%)		
	C	H	Empirical formula
Acacia catechu tannin	54·2	5·5	—
Catechin autoxidation polymer	53·0	4·7	$C_{15}H_{10}O_6,3H_2O$
Uncaria gambir tannin	52·0	5·7	—

solvents (Bate-Smith, 1949) from fisetin and quercetagetin (Table 2). Authentic quercetagetin was isolated from *Tagetes erecta* flowers for the purpose of comparison.

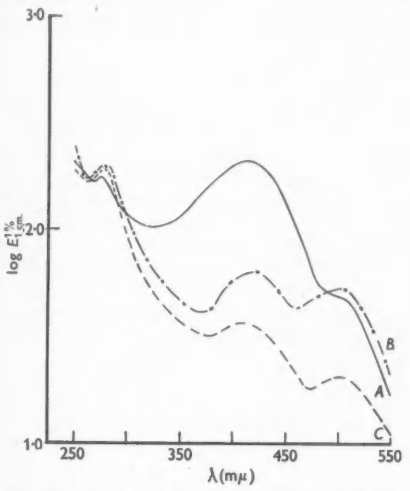


Fig. 6. Absorption spectra of catechin autoxidation polymer (*A*), and of tannins from *Acacia catechu* (*B*) and *Uncaria gambir* (*C*) in 0·07 M-phosphate buffer, pH 8.

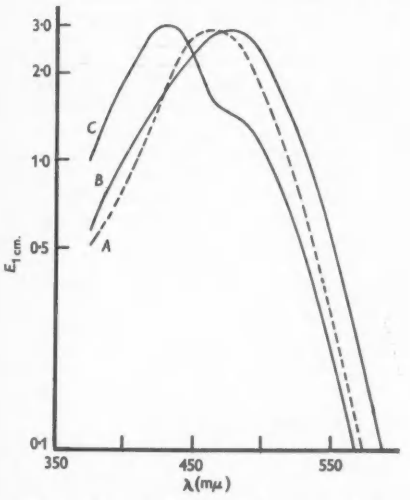


Fig. 7. Absorption spectra of the polymers resulting from aerobic oxidation of *homo*catechol and phloroglucinol (*A*), *homo*catechol and 5-methoxy-4-methylresorcinol (*B*), and catechin (*C*), by polyphenoloxidase.

DISCUSSION

A study of the course of aerobic oxidation of catechin by different plant polyphenoloxidases suggests that this substance functions as a substrate for these enzymes, which are widely distributed throughout plants. Both those polyphenoloxidases which are associated with the mitochondria, such as mushroom enzyme (Mason, 1955), and those which are not localized within the cell, such as tobacco enzyme (McClendon, 1953), have a common pattern of behaviour.

The products of polyphenoloxidase oxidation have been shown to be precisely similar to the polymer of catechin autoxidation which was formed by the quinone-polymerization mechanism. Additional evidence for the head-to-tail polymerization of catechin was also obtained from the aerobic oxidation of a mixture of *homo*catechol and 5-methoxy-4-methylresorcinol by polyphenoloxidase to a polymer which showed the characteristic absorption of the polymers of catechin oxidation. 4-Methyl-*o*-benzoquinone and 5-methoxy-4-methylresorcinol therefore undergo oxidative coupling. Aerobic oxidation of catechin by plant polyphenoloxidases proceeds by a quinone-polymerization mechanism and affords a product, the head-to-tail polymer units of which account for the profound difference in spectrum of the polymers from those of catechol polymers (Hathway & Seakins, 1957*a*). The fact that 4:5-dimethylcatechol does not undergo oxidative coupling with 5-methoxy-4-methyl-

Table 2. *Chromatographic behaviour of flavonols*

Solvent system *A*: *m*-cresol–acetic acid–water (50:2:48, by vol.; upper phase); solvent system *B*: butan-2-ol–acetic acid–water (14:1:5, by vol. v/v). Values given in parentheses were obtained with known compounds, the remainder with compounds obtained from *Acacia catechu*.

Compound	R_F *A*	*B*
Fisetin	0·37 (0·38)	0·72 (0·75)
Quercetin	0·23 (0·23)	0·82 (0·83)
Robinetin	— (0·19)	— (0·61)
Quercetagetin	0·13 (0·13)	0·42 (0·44)
Myricetin	— (0·09)	— (0·57)

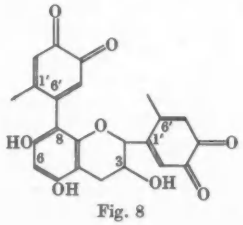


Fig. 8

resorcinol suggests that in these head-to-tail polymer units the 6′-position of one catechin-*o*-quinone residue is linked by a CC bond to the 6- or 8-position of another catechin-*o*-quinone residue. A partial type formula is shown in Fig. 8.

The polymer obtained by the enzymic oxidation of catechin has the analytical properties of a tannin (Gnamm, 1949; Schmidt, 1955). The polymer is precipitated from solution by gelatin or on refluxing with formalin–hydrochloric acid mixture, and it is retained by hide-powder (Grassmann *et al.* 1951). The failure of this polymer to give the vanillin reaction (Procter & Paessler, 1901) provides further evidence that polymerization involves the phloroglucinol residue of the monomer. The work of the Heidelberg School for 20 years has maintained (Freudenberg, 1956; Freudenberg & Maitland, 1934*a*, *b*; Freudenberg & Weinges, 1955) that phlobatannins from the barks of *Acacia mollissima*, birch, chestnut (*Castanea sativa*), eucalypts, mangrove, oak, spruce and willow and from the heartwood of *Acacia catechu* and quebracho trees are catechins, polymerized *post mortem* by plant acids. Two mechanisms have been considered. According to the first (Freudenberg & Maitland, 1934*a*, *b*), ring fission gives a secondary benzyl alcohol, and carbon atom $C_{(2)}$ then condenses either with carbon atom $C_{(6)}$ or with $C_{(8)}$ of another molecule to afford a bifunctional dimer, capable of further polymerization. More recently, Freudenberg (1956) has suggested that catechin may react as a pair of tautomeric diphenylpropenes which are involved in the first stage of a styrene polymerization (Freudenberg & Ahlhaus, 1956).

Phlobatannins have now been isolated in high yield from the tannin extractives of *Uncaria gambir* leaves and *Acacia catechu* heartwood, which are known to contain large quantities of catechin and its epimeride respectively. Since these substances have identical tannin properties, similar absorption spectra and elementary analyses to the polymer produced by polyphenoloxidase oxidation of catechin, a close structural relationship exists, and it is probable that these phlobatannins result from similar aerobic oxidation of catechin precursors. Formation of phlobatannins from catechin epimers by quinone polymerization recalls the similar formation of artificial melanins from 5:6-dihydroxyindoles (Beer *et al.* 1954; Bu'Lock & Harley-Mason, 1951), but it was not known whether the products resembled melanins of animal or plant origin (A. Robertson, personal communication). The fact that these new phlobatannins may be accounted for by catechin polymerization is in agreement with Freudenberg & Maitland's (1934*a*, *b*) suggestion, but no evidence has been found for the acid-catalysed reaction, which requires low pH (<2) and high temperature ($>50°$). The remaining phlobatannins from *Acacia catechu* and *Uncaria gambir* were shown by two-dimensional paper chromatography to contain mixtures of mobile substances. The absorption spectra of the crude mixtures were similar to those of the catechin oxidation polymers, but solubility in solvents and mobility in chromatographic-solvent systems implied a lower degree of polymerization.

Unlike the *Acacia catechu* and *Uncaria gambir* phlobatannins, a phlobatannin was isolated during the course of this work from a commercial extract derived from *Acacia mollissima* bark, which gave a vanillin reaction, a strong-violet ferric reaction, and a different absorption spectrum. It is therefore inadmissible to regard the phlobatannins at present as members of a closely related chemical family, but the presence of catechin derivatives in the cacao bean (Forsyth, 1952*a*) and tea leaf (Roberts, 1952), and of leucoanthocyanins in the bark of various eucalypts (Hillis, 1954, 1956) may indicate that the phlobatannins arise through quinone polymerization.

The present conclusion that enzymic formation of *Uncaria gambir* and *Acacia catechu* phlobatannins occurs in the detached leaves and heartwood respectively connects the formation of these compounds with the plant-browning reaction (Szent-Györgi & Vietorisz, 1931).

SUMMARY

1. Catechin was oxidized aerobically by mushroom, potato and tobacco polyphenoloxidases.

2. Catechin autoxidation and polyphenoloxidase oxidation polymers analyse as $C_{15}H_{10}O_6,3H_2O$, exhibit absorption bands at 270 and 410 mμ, and a shoulder at 500 mμ, and have identical analytical properties.

3. Aerobic oxidation of mixed synthetic substrates, such as *homo*catechol and 5-methoxy-4-methylresorcinol by polyphenoloxidase, gave a polymer showing the characteristic absorption of the polymers of catechin oxidation.

4. Aerobic oxidation of catechin by polyphenoloxidase therefore involves quinone polymerization.

5. Phlobatannins have been isolated in high yield from the extractives of harvested *Uncaria gambir* leaves and *Acacia catechu* heartwood, which have the same analytical properties as and similar elementary analyses and absorption spectra to the polymers of catechin oxidation.

6. The conclusion is drawn that these phlobatannins are formed by aerobic oxidation of catechin epimers by polyphenoloxidases in the detached leaves and heartwood respectively.

7. A flavonoid fraction from *A. catechu* contained three flavonols, chromatographically indistinguishable from fisetin, quercetagetin and quercetin.

The authors wish to thank Dr H. Phillips for his interest, and the Council of the British Leather Manufacturers' Research Association for their permission to publish this paper. *Acacia catechu* heartwood and harvested leaves of *Uncaria gambir* were obtained through the good offices of the State Silviculturist, Ootacamund Post, The Nilgiris, Madras State, India. We are also indebted to Dr E. C. Bate-Smith, Low Temperature Research Station, Cambridge, for a specimen of fisetin, to Professor D. Keilin, F.R.S., The Molteno Institute of Biology and Parasitology, Cambridge, for purified horse-radish peroxidase, and to Mr W. W. Reid, F.R.I.C., of Carreras Ltd., London, N.W. 1, for the acetone-dried powder of *Nicotiana tabacum*.

REFERENCES

Bate-Smith, E. C. (1949). *Symp. biochem. Soc.* no. **3**, p. 62.
Beer, R. J. S., Broadhurst, T. & Robertson, A. (1954). *J. chem. Soc.* p. 1947.
Bu'Lock, J. D. & Harley-Mason, J. (1951). *J. chem. Soc.* p. 703.
Erdtman, H. (1956). *Sci. Proc. R. Dublin Soc.* **27**, 129.
Forsyth, W. G. C. (1952*a*). *Biochem. J.* **51**, 511.
Forsyth, W. G. C. (1952*b*). *Biochem. J.* **51**, 516.
Freudenberg, K. (1956). *Sci. Proc. R. Dublin Soc.* **27**, 153.
Freudenberg, K. & Ahlhaus, O. (1956). *Mh. Chem.* **87**, 1.
Freudenberg, K. & Maitland, P. (1934*a*). *Liebigs Ann.* **510**, 193.
Freudenberg, K. & Maitland, P. (1934*b*). *Collegium, Haltingen*, **776**, 656.
Freudenberg, K. & Weinges, K. (1955). *Liebigs Ann.* **590**, 140.
Gnamm, H. (1949). In *Die Gerbstoffe und Gerbmittel*, 3rd ed. p. 55. Stuttgart: Wissenschaftliche Verlag.
Goris, M. (1907). *C.R. Acad. Sci., Paris*, **144**, 1162.
Grassmann, W., Endisch, O. & Kuntara, W. (1951). *Das Leder*, **2**, 202.
Graubard, M. & Nelson, J. M. (1935). *J. biol. Chem.* **111**, 757; **112**, 135.
Haldane, J. B. S. (1921). *J. Path. Bact.* **23**, 443.
Hathway, D. E. & Seakins, J. W. T. (1955). *Nature, Lond.*, **176**, 218.
Hathway, D. E. & Seakins, J. W. T. (1957*a*). *J. chem. Soc.* p. 1562.
Hathway, D. E. & Seakins, J. W. T. (1957*b*). *Biochem. J.* **65**, 32P.
Hillis, W. E. (1954). *J. Soc. Leath. Tr. Chem.* **38**, 91.
Hillis, W. E. (1956). *Aust. J. biol. Sci.* **9**, 263.
Jaccard, P. (1938). *Ber. eidgenöss. Techn. Hochsch. Zurich.* no. 36.
Keilin, D. & Mann, T. (1938). *Proc. Roy. Soc.* B, **125**, 187.
Lovering, P. E. & Smith, M. L. (1946). *Chem. & Ind.* p. 298.
McClendon, J. H. (1953). *Amer. J. Bot.* **40**, 260.
Mahal, H. S. (1938). *J. Indian chem. Soc.* **15**, 87.
Mason, H. S. (1955). *Advanc. Enzymol.* **16**, 152.
Perkin, A. G. (1897). *J. chem. Soc.* **71**, 1136.
Procter, H. R. & Paessler, J. (1901). In *Leitfaden für Gerbereichemische Untersuchungen*, p. 78. Berlin: Julius Springer.
Raper, H. S. (1932). *Ergebn. Enzymforsch.* **1**, 270.
Raper, H. S. & Wormall, A. (1923). *Biochem. J.* **17**, 457.
Reid, W. W. (1956). *Symp. Soc. Leath. Tr. Chem.* (Vegetable Tannins) p. 75.
Roberts, E. A. H. (1952). *J. Sci. Fd Agric.* **5**, 193.
Roberts, E. A. H. & Wood, D. J. (1950). *Biochem. J.* **47**, 175.
Robertson, A. & Whalley, W. B. (1951). *J. chem. Soc.* p. 3355.
Schmidt, O. T. (1955). In *Modern Methoden der Pflanzenanalyse*, vol. 3, p. 515. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
Szent-Györgi, A. & Vietorisz, K. (1931). *Biochem. Z.* **233**, 236.

The Characteristics of Hexokinase from *Locusta migratoria* Muscle

By MARGARET KERLY and D. H. LEABACK*
Department of Biochemistry, University College London

(*Received* 5 *February* 1957)

Hexokinases from different tissues vary in their properties and differ in particular in specificity towards their sugar substrates. Those enzymes which phosphorylate a number of sugars, including both glucose and fructose, have been described conveniently by Colowick (1951) as 'non-specific', whilst Medina & Sols (1956) suggest that the term ketokinase should be used for those which attack more than one ketose, but not aldoses, and that the name fructokinase be reserved for enzymes which act only with fructose. Non-specific hexokinases have been shown to be present in yeast (Berger, Slein, Colowick & Cori, 1946; Kunitz & McDonald, 1946), in higher plants (Saltman, 1953) and in a number of animal tissues (e.g. brain, Slein, Cori & Cori, 1950; retina, Hoare & Kerly, 1954; intestine, Sols, 1956). The specificity of the enzyme in heart muscle is similar to that of the enzyme in brain (Crane & Sols, 1955), but more than one enzyme may occur in mammalian skeletal muscle. Crane & Sols (1955) describe preparations of the non-specific enzyme from rat and dog muscle and Meyerhof (1927), in his early experiments on rabbit-muscle extracts, found that lactic acid was produced from glucose, mannose and fructose. Slein *et al.* (1950) showed that similar crude extracts could be fractionated to yield a partially purified enzyme which phosphorylated fructose but had little or no

* Present address: Institute of Orthopaedics, Stanmore, Middlesex.

activity towards glucose. This enzyme, like the ketokinase from liver, forms fructose 1-phosphate (Leuthardt & Testa, 1951; Hers, 1953). The occurrence of a hexokinase in insect tissue has been demonstrated by Chefurka (1954), who used homogenates of whole house-flies, and the presence in locust muscle of an enzyme which phosphorylates glucose can be inferred from the work of Rees (1954). The experiments described in this report provide evidence that the hexokinase in locust muscle is of the non-specific type and is not associated with a ketokinase.

EXPERIMENTAL

Materials. Adenosine triphosphate (ATP) was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). It was further purified (after decomposition of the mercury salt) by passage of the cold solution through a column of Amberlite IR 100 in the acid form. The sugars used (commercial preparations, AnalaR when available) were not further purified except for glucosamine hydrochloride, which was recrystallized twice from methanol–water (1:1). Glucose and fructose 6-phosphates (G6-P and F6-P) and inosine triphosphate (ITP) were prepared in this Department and were presented to us by Dr Datta and by Dr Hoare respectively. Glass-distilled water was used for the preparation of all solutions.

Preparation of muscle extracts. Adult male *Locusta migratoria* from the Anti-Locust Research Centre, London, were fed freshly cut grass but were starved for 72 hr. before use, as preliminary experiments (see below) showed this to be advisable. Immediately before dissection the locusts were inactivated by cooling to 5° for a short time. Since it is difficult to dissect out locust muscle in quantity a procedure based on that of Rees (1954) was adopted. The head, abdomen and appendages were removed with scissors and the thoraces cut down the mid-dorsal line; after removal of the intestine and most of the salivary gland, the preparations were placed in ice-cold 0·9% (w/v) NaCl soln. until a sufficient number (up to 48) had been dissected. Each thorax was blotted and after removal of the remaining traces of salivary gland placed on a chilled watch glass and finely chopped with scissors. The mass of tissue was weighed and ground vigorously for 4 min. in a glass mortar, with gradual addition of 5 vol. of water. After adjustment of the pH to 7·5 (Johnson's short-range indicator paper) with 2N-NaOH soln. the extract was squeezed through muslin into a chilled centrifuge tube. The supernatant formed after spinning at 0° for 30 min. at 18000 *g*, described as the extract, was used as the source of hexokinase. The protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). In preliminary tests, instead of being ground in a mortar the thoracic preparations were homogenized, either in water or in 0·2M-sucrose soln., and centrifuged at 600 *g*; the supernatant from such preparations is described as a homogenate.

Hexokinase assay. Muscle extracts were incubated with ATP and the appropriate sugar substrate, and the sugar remaining at the end of the incubation period was measured. Aldoses were determined by Somogyi's method (1952) and fructose by that of Bacon & Bell (1948).

The constituents of the medium shown below were pipetted into centrifuge tubes in a bath at 30°. At zero time 0·4 ml. of extract, pre-warmed to 30°, was added to each tube and after 15 min. the reaction was stopped by addition of 2 ml. each of 0·3M-$ZnSO_4$ soln. and 0·3N-$Ba(OH)_2$ soln. and 5 ml. of water. The tubes were centrifuged and sugar was estimated in the supernatant solution. Control tubes contained all solutions except ATP. The final concentration (mM) of the incubation mixture was: veronal buffer (pH 7·9), 10; hexose, 2·77; $MgCl_2$, 5; ATP, 5; total volume 1 ml.

In preliminary experiments in which homogenates were used NaF was also added to the incubation mixture; the volume of the mixture before addition of homogenate was then 0·5 and 0·1 ml. of 0·5M-NaF soln. was added 30 sec. after addition of the homogenate. Conditions were otherwise similar to those used for hexokinase assay in extracts.

Identification of reducing substances. In preliminary experiments (see below) when fed animals were used, reducing substances which were formed during incubation were identified chromatographically. Extracts were incubated with veronal buffer, pH 7·9, and 5 mM-$MgCl_2$ soln. for 2 hr., either alone or with addition of glycogen (1·67 mg./ml.); the reaction was stopped and the mixture deproteinized by addition of cold perchloric acid to give a final concentration of 5% (w/v). After neutralization with KOH soln. the precipitate was filtered off and the filtrate passed through a small column of Biodeminrolit resin (The Permitit Co. Ltd., London, W. 4) for removal of anions and cations and then freeze-dried. The residue was dissolved in a small volume of water, chromatographed on Whatman no. 1 paper by downward displacement with either *n*-butanol–acetic acid–water (4:1:5, v/v) or *n*-propanol–ethyl acetate–water (7:1:2, v/v), and developed by the benzidine reagent of Bacon & Edelman (1951).

RESULTS

Preliminary experiments

In early experiments with insects which had not been starved the reducing value of the mixture often, but not invariably, increased during incubation, making estimation of hexokinase ativity by determination of the amount of sugar removed impossible. If glycogen were added as well as glucose and ATP, additional reducing substances were always formed, which suggested that they were produced by hydrolysis of glycogen present in variable amount in the extracts. When the extracts were incubated without addition of glucose or ATP and the mixture was analysed chromatographically, glucose, maltose and reducing oligosaccharides were identified whether or not glycogen had been added before incubation, indicating that amylase was present in the extracts together with sufficient glycogen to produce enough reducing products for chromatographic detection, although not always sufficient for estimation by chemical analysis. Extracts prepared from locusts which had been starved for 72 hr. never produced reducing substances detectable by the Somogyi estimation when incubated without added glycogen, and the starvation procedure was adopted as a routine.

Table 1. *Hexokinase and amylase activity of homogenates*

Homogenate (0·4 ml.) was incubated for 15 min. at 30° in the media described in the text with glucose or fructose (for detection of hexokinase activity) or with glycogen (for detection of amylase activity).

Tissue	Protein content (mg./ml.)	Hexokinase activity (μmoles of sugar utilized)		Amylase activity (appearance of reducing products)
		Glucose	Fructose	
Thoracic muscle	5·3	2·72	1·39	+
Salivary gland	10·1	0·97	0·39	+
Exoskeleton	2·1	0·08	0	−

Table 2. *Comparison of hexokinase activity of homogenate and extract*

Homogenate is the supernatant after centrifuging at 600 *g* and extract is supernatant after centrifuging homogenate for 30 min. at 18 000 *g*. Homogenate or extract (0·4 ml.) was incubated with the medium described in the text for 15 min. at 30° with or without NaF.

	Protein content (mg./ml.)	Hexokinase activity (μmoles of glucose utilized)
Homogenate with 0·05 M-NaF	3·1	1·84
Extract with 0·05 M-NaF	1·8	1·80
Extract without NaF	1·8	2·03

Extracts prepared by Rees's (1954) method contained exoskeleton and salivary gland in addition to muscle; therefore the hexokinase and amylolytic activity of homogenates of exoskeleton, salivary gland and carefully dissected muscle were compared (Table 1). No significant activity was detected in the homogenate from the exoskeleton but that from the salivary gland showed appreciable hexokinase and amylolytic activity, though much less, especially if the protein content of the two homogenates is taken into account, than that of the muscle homogenate. In later experiments care was taken in preparation of extracts to remove all traces of salivary gland.

Comparison of the activity of homogenates and extracts from the same preparations (Table 2) showed that, unlike the calf-brain enzyme studied by Crane & Sols (1953), the locust hexokinase was not sedimented by centrifuging at 18 000 *g*. It was considered preferable to use extracts rather than homogenates since centrifuging at high speed removed nearly half the protein present in the homogenate and because experiment showed that it was not necessary to add fluoride when estimating hexokinase activity in extracts (see Table 2).

Activity was not diminished by incubating extracts for 1 hr. at 30°, storing for 24 hr. at 5°, for 3 months at −10° or by freeze-drying. Dialysis for 5 hr. reduced activity somewhat and after dialysis for 18 hr. extracts were completely inactive. About half the original activity could be restored to extracts dialysed for 18 hr. by addition of Mg^{2+} or Mn^{2+} ions; Co^{2+} ions were less effective (Table 3) and Ca^{2+}, Zn^{2+}, Cu^{2+} and Be^{2+} ions were without effect. Recent studies (e.g. Hers, 1952) have shown that the phosphate donor for hexokinase may be a 1:1 complex of Mg^{2+} ions and ATP, since activity is greatest when these compounds are present in the

Table 3. *Effect of addition of metal ions on the hexokinase activity of extract dialysed for* 18 *hr.*

Extract (0·4 ml.) was incubated for 30 min. at 30°, with the standard medium from which Mg^{2+} ions were omitted; metal ions were substituted to give a concentration of 5 mM.

Added ion	Hexokinase activity (μmoles of glucose utilized)
None	0
Mg^{2+}	1·76
Mn^{2+}	1·31
Co^{2+}	0·35

Table 4. *Effect of concentration of adenosine triphosphate and of* Mg^{2+} *ions on the hexokinase activity of extract dialysed for* 18 *hr.*

Extract (0·4 ml.) was incubated for 30 min. at 30°; concentrations of ATP and of Mg^{2+} ions were as shown. The medium contained 10 mM-veronal buffer, pH 7·9, and 2·77 mM-glucose.

Concn. of ATP (mM)	Hexokinase activity (μmoles of glucose utilized)	
	5 mM-Mg^{2+}	10 mM-Mg^{2+}
2·5	0·88	0·89
5·0	1·31	1·31
7·5	1·02	1·34
10·0	0·97	1·44
20·0	0·57	1·16

Table 5. *Effect of change of* pH *on the hexokinase activity of extract*

Extract (0·4 ml.) was incubated for 15 min. at 30° with the medium described in the text, except that the pH of veronal buffer was varied as shown.

pH	Hexokinase activity (μmoles of glucose utilized)
4·9	0·71
5·8	0·99
7·0	1·06
7·9	1·32
8·6	1·39

Table 6. *Relative rate of utilization of hexoses by different extracts*

Extract (0·4 ml.) was incubated for 15 min. at 30° with the medium described in the text.

Extract no.	Hexokinase activity (μmoles of hexose utilized) with added			Ratio of activity glucose:fructose
	Glucose	Mannose	Fructose	
8	2·01	—	0·57	3·5
9	1·37	—	0·47	2·9
14	1·72	—	0·64	2·9
15	2·50	1·92	0·67	3·7
16	1·78	—	0·70	2·3

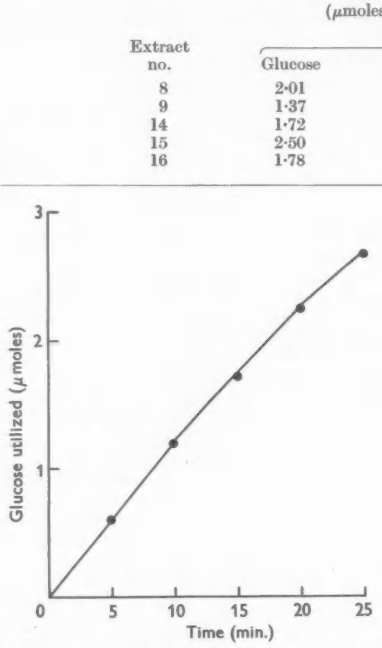


Fig. 1. Rate of utilization of glucose. Extract (0·4 ml.) was incubated at 30° in the medium described in the text.

incubation medium in equimolecular proportion. This was true for locust-muscle extracts (Table 4). No phosphorylation occurred if ATP were replaced by ITP. Like other hexokinases the locust enzyme was most active at and above pH 7·9 (Table 5). Attempts to purify the enzyme by precipitation with $(NH_4)_2SO_4$ or ethanol or by the isoelectric method of Colowick (1951) were unsuccessful, as activity was destroyed by these treatments.

Sugars utilized

D-Glucose, D-mannose, D-fructose and D-glucosamine were utilized by locust-muscle extracts but not D-galactose, L-sorbose, D-ribose, D-arabinose or *N*-acetyl-D-glucosamine. Since ATP was necessary for utilization it is assumed that phosphorylation occurred. The relative rates at which glucose and fructose were utilized varied from one extract to another (Table 6). The rate of reaction with glucose as substrate was linear at first but later decreased somewhat (Fig. 1); it was not proportional to the amount of enzyme added (Table 7) and fell off more

Table 7. *Effect of enzyme concentration on hexokinase activity*

Varying amounts of extract were incubated in the standard medium (final vol. 1 ml.) for 15 min. at 30°.

Extract added (ml.)	Hexokinase activity (μmoles of glucose utilized)
0·4	2·5
0·3	1·5
0·2	0·7
0·1	0·1

Table 8. *Effect of addition of hexoses on the utilization of fructose*

Extract (0·4 ml.) was incubated with 2·77 mM-fructose for 15 min. at 30° in the medium described in the text.

Hexose added	Concn. of hexose added (mM)	Hexokinase activity (μmoles of fructose utilized)	
		Extract *A*	Extract *B*
None		0·76	0·66
Glucose	1·39	0·20	—
	2·77	0·04	—
	5·54	0	—
Mannose	1·39	0·16	—
	2·77	0·05	—
	5·54	0	—
Galactose	2·77	0·72	—
N-Acetyl-D-glucosamine	1·0	—	0·26
	2·0	—	0·22
	3·0	—	0·15
	4·0	—	0·07
	5·0	—	0

rapidly than the concentration; a similar effect has been observed with rat-muscle extracts (Gerritsen, 1952; Liébecq, 1952).

Phosphorylation of fructose was strongly inhibited by the presence of glucose or mannose but not by that of galactose; *N*-acetyl-D-glucosamine, although not itself attacked by the enzyme, also inhibited phosphorylation (Table 8). Phosphorylation of both glucose and fructose was completely inhibited by addition of either G6-P or F6-P at

a concentration of 5 mM. In this respect the locust enzyme resembles that from brain but not that from yeast (Crane & Sols, 1953).

Like other hexokinases which catalyse phosphorylation of glucose and mannose, the locust enzyme had a high affinity for these sugars, being saturated at a concentration of 2·77 mM. In contrast the rate of fructose phosphorylation was dependent on the initial concentration (Table 9). The Michaelis constant for fructose was calculated by the method of Lineweaver & Burk (1934) from the data given in Table 9 and was found to be approx. 5·5 mM. The low affinity for fructose was not due to too low a concentration of ATP since increase in the concentration of ATP and of Mg^{2+} ions during incubation did not affect the rate of phosphorylation (Table 9, extract 20).

Phosphorylation product

The difference of affinity of the enzyme for glucose and fructose and variation in the relative rate of phosphorylation of the two sugars from extract to extract might have been due to the presence of a ketokinase. Had this been so, on analogy with the enzyme from liver and rabbit muscle, the reaction product would have been fructose 1-phosphate (F 1-P), not F 6-P, the product formed by the non-specific hexokinases. The two esters can be readily distinguished by acid hydrolysis; therefore the ester formed during incubation of fructose with a homogenate dialysed for 5 hr. (which contained negligible amounts of esterified phosphate) was isolated as the barium salt by a method similar to that of Leuthardt & Testa (1951), and its rate of hydrolysis determined. The reaction was stopped and the solution deproteinized by addition of an equal volume of 10% (w/v) trichloroacetic acid. A barium salt, insoluble in acetone but soluble in water, was isolated and dissolved in N-HCl; the barium was removed with Na_2SO_4 and the free ester analysed.

The ketose content corresponded to approximately 18% of the hexose equivalent of the total reducing value, whereas the phosphate content was somewhat greater than that to be expected for a monophosphate, which suggested that the ester was a mixture of glucose and fructose phosphates possibly contaminated with some diphosphate. On hydrolysis in N-HCl at 100° phosphate was liberated at a rate to be expected from a slowly hydrolysed hexose 6-phosphate, but there was no evidence of the presence of the rapidly hydrolysed F 1-P (Fig. 2).

Table 9. *Effect of concentration of fructose on hexokinase activity*

Extract (0·4 ml.) was incubated for 15 min. at 30° in the medium described in the text.

Concn. of fructose (mM)	Hexokinase activity (μmoles of fructose utilized)			
Extract no. ...	8	14	15	20*
1·39	0·35	0·45	0·38	0·34
2·77	0·57	0·64	0·67	0·62
5·54	0·88	1·05	0·82	1·01
11·08	1·13	1·38	1·13	1·18
K_m (mM)	6·2	5·8	3·7	6·3
Average	5·5			

* Concn. of both ATP and Mg^{2+} ions was increased to 10 mM.

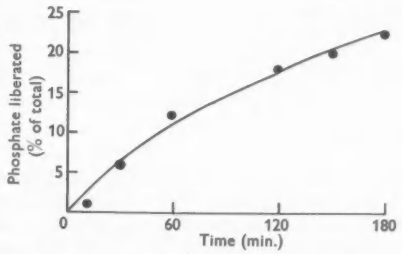


Fig. 2. Rate of hydrolysis in N-HCl of hexose phosphate isolated after incubation of extract with fructose (see text for details).

DISCUSSION

The substrates attacked by extracts of locust muscle, D-glucose, D-mannose, D-fructose and D-glucosamine are those phosphorylated by the non-specific hexokinase found in yeast, brain and other tissues. The isolation, as a product of fructose phosphorylation, of an ester hydrolysable only slowly by acid and having a higher reducing value than that to be expected from the ketose content, indicated the formation of a mixture of G 6-P and F 6-P and, assuming isomerase activity to be low, is evidence against the presence of a ketokinase. Further support for the non-occurrence of a ketokinase is afforded by the finding that fructose phosphorylation is inhibited by glucose and mannose and especially by *N*-acetylglucosamine, which is itself not phosphorylated (see Harpur & Quastel, 1949). The variation in the relative activity of different locust-muscle preparations towards glucose and fructose cannot be regarded as evidence for the occurrence of two different enzymes, as in crude extracts too many factors can affect the relative rates of hexose phosphorylation to make this a criterion of hexokinase specificity (cf. Wiebelhaus & Lardy, 1949). Slein *et al.* (1950) consider the low affinity of ketokinase for fructose to be characteristic of this enzyme, but this seems unlikely since the Michaelis constants reported for

the ketokinases from different sources differ so widely (liver $<0{\cdot}5$ mM and rabbit muscle $0{\cdot}2$ M; Hers, 1955). The constant calculated for the locust-muscle enzyme, 5·5 mM, is of the same order as that found for hexokinase from yeast, 1·7 mM, by Slein *et al.* (1950).

We consider that locust muscle contains a single, non-specific hexokinase and that the enzyme is similar to that found in yeast and in brain in its general characteristics. In contrast to hexokinase from brain the locust enzyme is not sedimented by centrifuging at 18000 *g* but, like the brain enzyme and in contrast to that from yeast, it is inhibited by the products of its action, G 6-P or F 6-P.

SUMMARY

1. Hexokinase activity has been demonstrated in extracts of thorax muscle and salivary gland of *Locusta migratoria*.

2. The characteristics of the enzyme are similar to those of the hexokinase present in brain extracts.

3. In the presence of adenosine triphosphate and Mg^{2+} ions, D-glucose, D-mannose, D-fructose and D-glucosamine are utilized.

4. Utilization of all substrates is inhibited by glucose 6-phosphate or fructose 6-phosphate, and that of fructose by glucose, mannose and *N*-acetylglucosamine.

5. An ester stable to acid hydrolysis, probably a mixture of glucose 6-phosphate and fructose 6-phosphate, has been identified as a product of phosphorylation of fructose.

6. Extracts of muscle and of salivary gland contain an amylase which hydrolyses glycogen present in extracts of fed insects.

We should like to thank Dr S. P. Datta for the gift of hexose phosphates, Dr D. S. Hoare for inosine triphosphate and the Director of the Anti-Locust Research Centre for locusts.

REFERENCES

Bacon, J. S. D. & Bell, D. J. (1948). *Biochem. J.* **42**, 397.
Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.
Chefurka, W. (1954). *Enzymologia*, **17**, 73.
Colowick, S. P. (1951). *The Enzymes*, vol. 2, p. 129. New York: Academic Press Inc.
Crane, R. K. & Sols, A. (1953). *J. biol. Chem.* **203**, 273.
Crane, R. K. & Sols, A. (1955). *Methods in Enzymology*, vol. 1, p. 277. New York: Academic Press Inc.
Dounce, A. L., Rothstein, A., Beyer, G. T., Meier, R. & Freer, R. M. (1948). *J. biol. Chem.* **174**, 361.
Gerritsen, T. (1952). *Biochim. biophys. Acta*, **8**, 466.
Harpur, R. P. & Quastel, J. H. (1949). *Nature, Lond.*, **164**, 693.
Hers, H. G. (1952). *Biochim. biophys. Acta*, **8**, 424.
Hers, H. G. (1953). *Arch. int. Physiol.* **61**, 426.
Hers, H. G. (1955). *Methods in Enzymology*, vol. 1, p. 286. New York: Academic Press Inc.
Hoare, D. S. & Kerly, M. (1954). *Biochem. J.* **58**, 88.
Kunitz, M. & McDonald, M. R. (1946). *J. gen. Physiol.* **29**, 393.
Leuthardt, F. & Testa, E. (1951). *Helv. chim. acta*, **34**, 931.
Liébecq, C. (1952). *Arch. int. Physiol.* **60**, 562.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Medina, A. & Sols, A. (1956). *Biochim. biophys. Acta*, **19**, 378.
Meyerhof, O. (1927). *Biochem. Z.* **183**, 176.
Rees, K. R. (1954). *Biochem. J.* **58**, 196.
Saltman, P. (1953). *J. biol. Chem.* **200**, 145.
Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
Sols, A. (1956). *Biochim. biophys. Acta*, **19**, 144.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Wiebelhaus, V. D. & Lardy, H. A. (1949). *Arch. Biochem.* **21**, 321.

The Hexose Specificity of Hexokinase in Brain from Various Species

By MARGARET KERLY and D. H. LEABACK*
Department of Biochemistry, University College London

(*Received* 5 *February* 1957)

Geiger (1940) was the first to demonstrate hexokinase activity in extracts of rat brain, and later workers have shown the enzyme to be present in ox (Colowick, Cori & Slein, 1947), sheep (Slein, Cori & Cori, 1950) and mouse (Hesselbach & DuBuy, 1953) brain. The studies of later workers [especially that of Sols & Crane (1954) on calf brain] indicate that the enzyme in mammalian brain resembles that from yeast in its ability to attack a number of sugars. The experiments described below show that an enzyme with a similar specificity is found in brain tissue of birds, fishes, amphibia and molluscs.

* Present address: Institute of Orthopaedics, Stanmore, Middlesex.

EXPERIMENTAL

Brain extracts were prepared by a method based on that of Weil-Malherbe & Bone (1951). Rats, pigeons, 4-day-old chickens and frogs were killed by decapitation or dislocation of the neck; the brain was removed immediately, placed in ice-cold 0·9% (w/v) NaCl and used at once for preparation of the extract. Brains were removed from marine animals at Plymouth and placed with ice in a vacuum flask for transit to London. Preliminary experiments with rat brain showed that this treatment caused no loss of activity. For preparation of the extracts one or more brains were removed from saline or ice, blotted and homogenized for 2 min. in 7·5 vol. of ice-cold water; the homogenate was centrifuged for 10 min. at 600 *g* at a temperature of 5°. The supernatant was poured off and the sediment suspended in a further 7·5 vol. of ice-cold water; it was centrifuged again and the supernatant added to that from the first centrifuging. Extracts prepared in this way were free from whole cells and large pieces of cell debris; the protein content was of the order of 6 mg./ml. Centrifuging of homogenates of pigeon brain at 18000 *g* for 30 min. caused sedimentation of about one-third of the enzyme. This is a smaller fraction than that reported by Crane & Sols (1953) for calf-brain and by Stern (1954) for rat-brain homogenates; possibly, as Stern has suggested, the amount of sedimentation depends on the technique used in preparation of the initial homogenate. In view of the results with pigeon brain, purification of homogenates from other species by high-speed centrifuging was not attempted. Hexokinase activity was assayed by estimation of the hexose utilized during incubation with adenosine triphosphate (ATP) and $MgCl_2$ as previously described (Kerly & Leaback, 1957).

RESULTS

Glucose and fructose were utilized at a high rate by extracts of all the brains examined (Table 1). There was no utilization without addition of ATP and it is concluded that the sugars were phosphorylated. The rate for glucose utilization was always greater than that for fructose but the ratio of glucose to fructose used varied from one homogenate to another. Phosphorylation of glucose was partially inhibited by the presence of an equimolecular concentration of fructose, but addition of glucose completely inhibited utilization of fructose. This suggested that the hexokinase present in the brains of these animals was, like that present in calf and rat brains, of the non-specific type which attacks both aldoses and ketoses. This view was supported by the results of further experiments in which the effect of alteration of substrate concentration on the utilization of fructose by pigeon- and rat-brain homogenate was compared (Table 2). The activity of the homogenates varied with the concentration of fructose, and from the data obtained the Michaelis constants for the enzymes from rat (1·7 mM) and pigeon (1·8 mM) brain were calculated by the method of Lineweaver & Burk (1934). The constants are similar to those found for the non-specific hexokinase in ox brain (1·6 mM, Sols & Crane, 1954) and in yeast (1·7 mM, Slein *et al.* 1950) and unlike those reported by Hers (1955) for the

Table 1. *Utilization of glucose and fructose by brain homogenates from different animals*

Homogenate (0·4 ml.) was incubated for 15 min. at 30° in a medium which contained veronal buffer (pH 7·9; 10 mM), $MgCl_2$ (5 mM), ATP (5 mM), NaF (50 mM) and hexose as shown. Total volume, 1 ml.

Substrate	Hexokinase activity: Glucose 2·77 mM (μmoles of glucose utilized)	Hexokinase activity: Glucose 2·77 mM + Fructose 2·77 mM* (μmoles of glucose utilized)	Hexokinase activity: Fructose 2·77 mM (μmoles of fructose utilized)
Mollusca			
Sepia officinalis (squid)	1·14	1·09	0·57
Elasmobranch			
Scyllium canicula	1·06	1·02	0·48
Raia brachyura	0·83	0·67	0·69
Teleost			
Scophthalmus maximus	1·10	0·97	0·38
Gadus merangulus	0·94	0·84	0·78
Amphibia			
Rana temporaria	1·08	0·56	0·40
Avies			
Columba (Pigeon)	1·54	1·42	0·58
Gallus (Chicken)	1·48	1·15	0·72
Mammalia			
Rat	1·16	1·11	0·48

* When glucose and fructose were both present, no fructose was utilized by any of the homogenates.

ketokinase in liver (<0·5 mM) and in rabbit muscle (0·2 M).

Further evidence for the occurrence of a single enzyme is given by the effect of addition of mannose, *N*-acetyl-D-glucosamine and galactose on fructose utilization (Table 3); the two former, like glucose, caused inhibition whereas the last had no action. Phosphorylation of both glucose and fructose by homogenates of pigeon and chick brain was prevented by addition of glucose or fructose 6-phosphates, the reaction products of the non-specific hexokinase. This also supports the suggestion that only one enzyme is present. In this respect the hexokinase in these brains resembles that found in ox and rat brain and differs from that found in yeast, which is not inhibited by its reaction product. We conclude from these findings that the hexokinase present in pigeon brain and probably also that present in the brains of the other species examined resembles the enzyme found in mammalian brain in its specificity towards hexoses.

Table 2. *Effect on fructose utilization by brain homogenates of variation of concentration of substrate*

Homogenate (0·4 ml.) was incubated for 15 min. at 30° in a medium which contained veronal buffer (pH 7·9; 10 mM), $MgCl_2$ (5 mM), ATP (5 mM), NaF (50 mM) and fructose as shown. Total volume, 1 ml.

Fructose concn. (mM)	Hexokinase activity (μmoles of fructose utilized)	
	Rat brain	Pigeon brain
1·39	0·74	0·50
2·77	1·11	0·67
5·54	1·32	0·82
11·08	1·39	0·92

Table 3. *Effect on fructose utilization by brain homogenates of addition of glucose, mannose, galactose and N-acetyl-D-glucosamine*

Homogenate (0·4 ml.) was incubated for 15 min. at 30° in a medium which contained veronal buffer (pH 7·9; 10 mM), $MgCl_2$ (5 mM), ATP (5 mM), NaF (50 mM) and fructose (2·77 mM). Total volume, 1 ml.

Concn. of added hexose (mM)		Hexokinase activity (μmoles of fructose utilized)	
		Rat brain	Pigeon brain
None		1·10	0·95
Glucose	1·39	0·14	0·37
	2·77	0	0
Mannose	1·39	0·27	0·14
	2·77	0	0
Galactose	2·77	1·06	0·97
N-Acetyl-D-glucosamine	1·00	0·52	0·60
	2·00	0·31	0·36
	5·00	0·05	0

SUMMARY

1. Extracts of brains from pigeon, 4-day-old chick, two elasmobranch and two teleost fishes, frog and squid phosphorylate glucose and fructose.

2. In pigeon-brain extracts phosphorylation of fructose is inhibited by mannose and by *N*-acetyl-D-glucosamine; phosphorylation of both glucose and fructose is inhibited by glucose and fructose 6-phosphates.

3. The affinity for fructose of the enzyme present in rat and pigeon brain is similar to that of calf-brain hexokinase.

We should like to thank the Director of the Marine Biological Association Laboratory, Plymouth, for assistance in obtaining marine material.

REFERENCES

Colowick, S. P., Cori, G. T. & Slein, M. W. (1947). *J. biol. Chem.* **168**, 583.
Crane, R. K. & Sols, A. (1953). *J. biol. Chem.* **203**, 273.
Geiger, A. (1940). *Biochem. J.* **34**, 465.
Hers, H. G. (1955). *Methods in Enzymology*, vol. 1, p. 286. New York: Academic Press Inc.
Hesselbach, M. L. & DuBuy, H. G. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 62.
Kerly, M. & Leaback, D. H. (1957). *Biochem. J.* **67**, 245.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
Sols, A. & Crane, R. K. (1954). *J. biol. Chem.* **210**, 581.
Stern, J. (1954). *Biochem. J.* **58**, 536.
Weil-Malherbe, H. & Bone, A. D. (1951). *Biochem. J.* **49**, 339.

The Identification of Aldoheptose Sugars

By D. A. L. DAVIES
Microbiological Research Establishment, Porton, Wilts

(*Received* 11 *March* 1957)

Ketoheptose sugars have long been known to occur in plants, both free and in phosphorylated form (references cited by MacLennan & Davies, 1957). Aldoheptose sugars have been encountered only during the last few years and have been found as constituents of polysaccharides isolated from Gram-negative bacteria. Only since simple methods have become available for the detection of heptose sugars has it become apparent that they are of frequent occurrence in these polysaccharides. It is also clear that in some instances they have been overlooked in the past, or incorrectly identified as one of the commonly occurring hexoses.

In only three cases have aldoheptose sugars been isolated from natural products and identified. From a *Shigella flexneri* polysaccharide, L-*glycero*-D-*manno*-heptose was obtained by Slein & Schnell (1953). The same sugar was isolated by Weidel (1955) from *Escherichia coli* cell walls. A *Chromobacterium violaceum* polysaccharide was described by Davies (1955) and D-*glycero*-D-*galacto*-heptose was subsequently isolated from the material (MacLennan & Davies, 1957).

Aldoheptoses have been detected in polysaccharides isolated from the following bacteria: *Shigella sonnei* (Jesaitis & Goebel, 1952), *E. coli* strains (Weidel, Koch & Bobosch, 1954; Fromme, Lüderitz & Westphal, 1954), *C. violaceum* strains (Davies, 1955), *Pasteurella pestis* (Davies, 1956), *P. pseudotuberculosis* and *P. septica* (Davies, 1957), *P. haemolytica*, *Neisseria catarrhalis*, *Haemophilus* spp., etc. (Crumpton, Davies & MacLennan, in preparation). In some, for example, *P. pestis*, the heptose is the major sugar constituent of the polysaccharide.

The simplest method of detection is the sulphuric acid–cysteine reaction described by Dische (1953, 1954), in which polysaccharides without prior hydrolysis give characteristic absorption curves due to their monosaccharide constituents; aldoheptoses give an absorption maximum at 505 mμ.

Paper chromatography can be used for detection; the anisidine-spray reagent of Hough, Jones & Wadman (1950) has been found to give a characteristic colour with aldoheptose sugars (greenish purple) which is quite different from that given by any other class of sugar. Provisional identification can also be made by this means, which is especially valuable when only small amounts of material are available, as is frequently the case with bacterial products. Unfortunately, the heptose sugars are not readily available for comparison with unidentified components. There are records of R values for a few aldoheptoses in the literature (Hirst & Jones, 1949; Isherwood & Jermyn, 1951) but the lack of data for the remainder of the sixteen configurations makes these of only limited value for identification.

In the course of examining the heptose constituents of a number of polysaccharides, twelve different aldoheptoses have been obtained through the generosity of donors acknowledged below. Some chromatographic data which have accumulated are presented in this communication in such a way that possible identifications may be made without using the known heptoses themselves as standards for comparison.

For comparison with isolated samples, infrared-absorption spectra of eight aldoheptoses have already been published (MacLennan & Davies, 1957).

MATERIALS AND METHODS

Sugars. Aldoheptoses and certain other sugars or derivatives from which solutions of the free sugar could be obtained were kindly supplied by Dr Richtmyer, National Institutes of Health, Bethesda, Md., U.S.A. and Dr Isbell, National Bureau of Standards, Washington, D.C., U.S.A. Acetyl derivatives of D-idose, D-*glycero*-D-*gluco*-heptose and D-*glycero*-D-*manno*-heptose were deacetylated with barium methoxide as described by Isbell (1930); D-*glycero*-D-*altro*-heptose was prepared from its aldonic acid lactone by reduction with sodium amalgam according to the method of Isbell (1953).

All of the common sugars were commercial samples; of the large number examined only a few have been found useful as index sugars.

Heptose detection. The H_2SO_4–cysteine reaction of Dische (1953) was used. To amounts of sugar in water (1 ml.), 4·5 ml. of H_2SO_4 (conc. A.R. H_2SO_4 diluted with one-sixth of its vol. of water) was added, tubes being agitated in a cooling bath during the addition of acid. Reaction mixtures were then heated at 100° for 3 min., cooled and 0·1 ml. of 3% cysteine hydrochloride was added. After mixing they were allowed to stand at 20°. For each amount of sugar three tubes were set up, cysteine being added to two of these tubes. Optical densities (D) were measured in a Unicam SP. 600 spectrophotometer after 22 hr., with a 1 cm. cell. Figures recorded are D_{505}–D_{540} with cysteine less D_{505}–D_{540} without cysteine, × 1000.

Chromatography. Single-dimensional descending paper chromatography was used with Whatman no. 1 paper and four solvent systems (basic, acidic, neutral and phenolic): butanol–pyridine–water (6:4:3), propan-1-ol–benzyl alcohol–formic acid as used by Giovannozzi-Sermanni (1956), 95% (v/v) acetone–water and phenol–aq. NH_3 soln. For detection anisidine hydrochloride (Hough *et al.* 1950) was used for reducing sugars and ammoniacal $AgNO_3$ soln. or periodate soln. (Wolfrom & Miller, 1956) for sugar alcohols. Sugars were applied to the paper in 100 μg. amounts in volumes of 10 μl.

EXPERIMENTAL AND RESULTS

Sulphuric acid–cysteine reaction

This has been fully described by Dische (1953). Different aldoheptoses give the same absorption peak but the intensity of absorption differs markedly in different cases. It can therefore be used for estimation only when the identity of a sugar is known. Estimations are reproducible and calibrations have not varied over a series of tests. The optical-density values follow a straight-line relationship with weight of sugar over the range 50–250 μg. Typical figures, obtained as described in Materials and Methods, for the two heptoses already isolated are: D-*glycero*-D-*galacto*-heptose 340–350 (100 μg.), 675–685 (200 μg.); D-*glycero*-L-*manno*-heptose 205–215 (100 μg.), 400–415 (200 μg.).

Paper chromatography

It is becoming generally accepted that R values alone are not sufficiently reliable for the identification of sugars on paper chromatograms. The order in which sugars separate, however, is wholly reproducible if an order can be established. It has not been possible to separate some of the slower-running heptoses in some solvents during the course of a single run, but much better results have been obtained by running a solvent to the bottom of the paper, drying thoroughly and running a second or even a third time (Jeanes, Wise & Dimler, 1951). By this method R values cannot be reproduced within sufficiently narrow limits to be of any real value, but a reliable order of separation can be obtained.

Table 1. *Separation of* D-*aldohexoses in different solvent systems on paper chromatograms, in order of increasing R value*

Butanol–pyridine–water	Propan-1-ol–benzyl alcohol–formic acid	Acetone–water	Phenol–aq. NH_3 soln.
Galactose	Galactose	Galactose	Glucose
Glucose	Glucose	Glucose	Gulose
Allose	Gulose	Mannose	Galactose
Gulose	Allose	Allose	Mannose
Mannose	Mannose	Gulose	Altrose
Altrose	Altrose	Altrose	Allose
Talose	Idose	Talose	Idose
Idose	Talose	Idose	Talose

The results of Isherwood & Jermyn (1951) showed that the order of separation in different classes of sugars is the same from the point of view of homomorphology. This has now been amplified for the aldoheptose series as compared with the aldohexoses and found to be true in a general way though not in detail. The order of separation of the aldohexoses is shown in Table 1.

By using the homomorphology correlation, and by interpolating readily available sugars or hexitols between the positions occupied by the aldoheptose sugars (data shown in Table 2) it is possible to deduce the probable identity of aldoheptoses without using the authentic sugar for comparison.

In order to obtain a sufficiently good separation, sugars were grouped into those which run faster or slower than glucose. Those running faster were separated in single runs when butanol–pyridine–water or aq. acetone were used and in double runs in phenol–aq. NH_3 soln. or propan-1-ol–benzyl alcohol–formic acid. Those running more slowly than glucose were separated in triple runs with the last solvent system and in double runs in the other three solvent systems.

Aldoheptoses generally run more slowly than aldohexoses but overlap the hexose region. In butanol–pyridine–water a number of interpolations were possible, in the other solvent systems fewer common sugars were available in the region of those aldoheptoses which ran more slowly than glucose.

In Table 2 R_g values are given only to indicate the region in which a sugar travels, and are not on a linear scale since the intervals between successive sugars are not shown.

DISCUSSION

The presence of an aldoheptose sugar in a polysaccharide can most easily be demonstrated by the sulphuric acid–cysteine reaction, but possible interference by pentoses should be allowed for (Dische, 1953). In most cases confirmation can be obtained by the appearance on paper chromatograms sprayed with anisidine hydrochloride of a characteristic greenish purple spot, although this can be obscured by the presence of other sugars. To obtain an indication of identity by using the data in Table 2 it is preferable to separate a heptose component in sufficient amount on a thicker paper for elution and re-running in the solvents described.

The 12 D-aldoheptoses for which positions are given include all of the C-1 to C-5 configurations; where both members of a pair have been used (-D-*gluco*- and -L-*gluco*-, -D-*manno*- and -L-*manno*-, -D-*galacto*- and -L-*galacto*-, -D-*talo*- and -L-*talo*-) it

can be seen that the members of pairs run adjacent to one another in most cases and have suffered only minor displacements in the remainder. Comparing Tables 1 and 2 it will also be seen that the order of separation of hexoses and heptoses do follow the homomorphology pattern in most cases, again with a few minor displacements. The positions given are the carefully checked results of a large number of runs in each solvent system, and it is considered that the regions in which the four missing aldoheptoses would lie could be judged with reasonable confidence; these four have yet to be synthesized (D-*glycero*-L-*gulo*-, D-*glycero*-L-*allo*-, D-*glycero*-L-*altro*- and D-*glycero*-L-*ido*-heptoses).

Table 2. *Order of separation of aldoheptoses and other sugars on paper chromatograms*

R_g values (distance travelled relative to glucose) are given only to indicate the region in which a sugar will be found and not as indices to the exact positions of any particular sugars. Sugars bracketed together do not separate significantly from one another.

R_g	Butanol–pyridine–water
0·6	Cellobiose
	D-Galactosamine, D-*Glycero*-L-*galacto*-heptose
	Maltose
	D-*Glycero*-D-*galacto*-heptose
	D-Glucosamine, D-*Glycero*-L-*gluco*-heptose
0·8	D-*Glycero*-D-*gluco*-heptose
	Galactitol, D-*Glycero*-D-*allo*-heptose, D-Galactose
	D-*Glycero*-D-*gulo*-heptose-
	D-Sorbitol
	D-*Glycero*-D-*manno*-heptose
	D-Mannitol, D-*Glycero*-L-*manno*-heptose
1·0	D-Glucose
	D-*Glycero*-L-*talo*-heptose
	D-*Glycero*-D-*altro*-heptose
	D-Allose
	L-Sorbose
	D-Mannose, L-Arabinose
	D-*Glycero*-D-*talo*-heptose
1·3	D-Xylose
	D-*Glycero*-D-*ido*-heptose, *N*-Acetyl-D-glucosamine
	D-Ribose
1·5	L-Rhamnose

R_g	Acetone–water
0·5	D-Glucosamine
	D-*Glycero*-L-*galacto*-heptose
	D-*Glycero*-D-*galacto*-heptose
	D-*Glycero*-L-*gluco*-heptose
	D-*Glycero*-D-*gluco*-heptose
	D-*Glycero*-L-*manno*-heptose
0·8	D-*Glycero*-D-*manno*-heptose
	D-*Glycero*-D-*gulo*-heptose
	D-*Glycero*-D-*allo*-heptose
	D-Galactose
1·0	D-Glucose
	D-Mannitol
	D-Sorbitol
	D-*Glycero*-L-*talo*-heptose
	D-*Glycero*-D-*altro*-heptose
	D-Mannose, D-*Glycero*-D-*talo*-heptose
1·5	L-Arabinose
2·0	D-*Glycero*-D-*ido*-heptose
	D-Xylose
	L-Fucose
	D-Lyxose

R_g	Phenol–aq. NH_3 soln.
0·8	D-*Glycero*-L-*gluco*-heptose, Melibiose
	D-*Glycero*-D-*gulo*-heptose, D-*Glycero*-D-*galacto*-heptose
0·9	D-*Glycero*-D-*gluco*-heptose
	Cellobiose
	Maltose
	D-*Glycero*-L-*galacto*-heptose
	D-*Glycero*-D-*manno*-heptose, D-*Glycero*-D-*altro*-heptose
1·0	D-Glucose
	D-*Glycero*-L-*manno*-heptose
	D-*Glycero*-D-*allo*-heptose, D-Gulose, L-Sorbose
	D-*Glycero*-D-*ido*-heptose, D-Galactose
	D-Xylose
1·2	D-*Glycero*-D-*talo*-heptose
	D-*Glycero*-L-*talo*-heptose, D-Lyxose
1·4	L-Arabinose
	D-Ribose

R_g	Propan-1-ol–benzyl alcohol–formic acid
0·4	Melibiose
	Cellobiose
	Maltose
	D-*Glycero*-L-*gluco*-heptose, D-*Glycero*-L-*galacto*-heptose, D-*Glycero*-D-*galacto*-heptose
	D-Glucosamine, D-Galactosamine
0·7	D-*Glycero*-D-*gluco*-heptose
	D-*Glycero*-D-*gulo*-heptose
	D-*Glycero*-D-*manno*-heptose
	D-*Glycero*-D-*allo*-heptose
	D-*Glycero*-L-*manno*-heptose
	D-Galactose
	D-*Glycero*-D-*altro*-heptose
1·0	D-Glucose
	D-*Glycero*-L-*talo*-heptose
	L-Sorbose, D-*Glycero*-D-*talo*-heptose
	D-Mannose
1·3	D-*Glycero*-D-*ido*-heptose
	L-Arabinose
1·6	D-Xylose

Thus an aldoheptose travelling between glucosamine and maltose in butanol–pyridine–water could be none other than D-*glycero*-D-*galacto*-heptose (or its optical enantiomorph L-*glycero*-L-*galacto*-heptose) since the missing heptoses could all be confidently expected to travel faster than this. Similarly, D-*glycero*-D-*talo*-heptose or its optical enantiomorph could be checked between different pairs of common sugars in each of the four solvent systems. By these methods a probable identification or a small number of alternatives could be deduced for any aldoheptose sugar.

Whereas the aldohexoses almost always occur in Nature in the D- form, the two aldoheptoses known thus far from natural sources are one D- and one L-. The determination of optical form can only be made on the isolated sugars or their derivatives.

SUMMARY

1. The behaviours of 12 of the 16 D-aldoheptose sugars have been recorded for four different solvent systems on paper chromatograms.

2. The positions of a number of common sugars have been determined in relation to the heptoses so that an idea of the identity of an unknown heptose may be gained without reference to authentic samples.

3. The order of separation of the eight D-aldohexoses and their homomorphous D-aldoheptoses are in tolerably good agreement.

I am greatly indebted to Dr N. K. Richtmyer and Dr H. S. Isbell for gifts of aldoheptose and other sugars. I wish also to thank Mr V. S. G. Baugh for valuable technical assistance.

REFERENCES

Davies, D. A. L. (1955). *Biochem. J.* **59**, 696.
Davies, D. A. L. (1956). *Biochem. J.* **63**, 105.
Davies, D. A. L. (1957). *J. gen. Microbiol.* (in the Press).
Dische, Z. (1953). *J. biol. Chem.* **204**, 983.
Dische, Z. (1954). *Methods of Biochemical Analysis*, vol. 2, p. 313. Ed. by Glick, D. New York: Interscience Publ. Inc.
Fromme, I., Lüderitz, O. & Westphal, O. (1954). *Z. Naturf.* B, **9**, 303.
Giovannozzi-Sermanni, G. (1956). *Nature, Lond.*, **177**, 586.
Hirst, E. L. & Jones, J. K. N. (1949). *Disc. Faraday Soc.* **7**, 268.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
Isbell, H. S. (1930). *J. Res. nat. Bur. Stand.* **5**, 1185.
Isbell, H. S. (1953). *J. Res. nat. Bur. Stand.* **50**, 133.
Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
Jesaitis, M. A. & Goebel, W. F. (1952). *J. exp. Med.* **96**, 409.
MacLennan, A. P. & Davies, D. A. L. (1957). *Biochem. J.* **66**, 562.
Slein, M. W. & Schnell, G. W. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 734.
Weidel, W. (1955). *Hoppe-Seyl. Z.* **299**, 253.
Weidel, W., Koch, G. & Bobosch, K. (1954). *Z. Naturf.* B, **9**, 575.
Wolfrom, M. L. & Miller, J. B. (1956). *Analyt. Chem.* **28**, 1037.

Heterogeneity of Normal Adult Human Haemoglobin

By A. C. ALLISON* and M. P. TOMBS†
Department of Biochemistry, University of Oxford

(*Received* 29 *April* 1957)

Studies with three different techniques have indicated that normal adult human haemoglobin is heterogeneous. These techniques are: solubility in variable solvents (Roche, Derrien & Roques, 1953*a*, *b*; Roche, Derrien, Reynaud, Laurent & Roques, 1954; Roche & Derrien, 1953); electrophoresis in starch slabs (Kunkel & Wallenius, 1955); and chromatography on columns of ion-exchange resins (Huisman & Prins, 1955; Morrison & Cook, 1955).

In the variable-solvent test a system is set up containing a constant amount of protein at a constant pH and varying concentrations of salts. The extent of precipitation of the protein or proteins depends upon the salt concentration in the way described by the Cohn equation, $\ln S = \beta - K(\Gamma/2)$, where S is the solubility in g./l., K is the salting-out constant, $\Gamma/2$ is the ionic strength of the precipitating salt and β is the extrapolated intercept of the linear portion of the curve on the ordinate ($\log S$) axis (Cohn, 1925). Using phosphate buffer, pH 6·5, as the precipitating salt, Roche and his colleagues (Roche *et al.* 1953*a*, *b*, 1954) described six breaks in the salting-out curve of normal adult carboxyhaemoglobin, and concluded that it contains three adult and three small foetal components. This interpretation has been criticized by Itano (1956), on the grounds that changes in the solid phase, which are

* Medical Research Council Unit for Research in Cell Metabolism.

† Supported by a research grant from the Agricultural Research Council.

reflected in discontinuities in the salting-out curve, might arise from alterations in the degree of aggregation of haemoglobin as a result of interaction with small molecules or ions in the solvent. This objection is partially overcome by the fractionation of three components in horse carboxyhaemoglobin (Roche *et al.* 1954). However, Ogston & Tombs (1956) have described a simple way of recognizing discontinuities due to changes in the solid phase and of distinguishing these from discontinuities due to the presence of more than one solute component. If two or more different species of protein are present in the solution, the position of the breaks will vary with the concentration of protein in solution at the start of the experiment. On the other hand, if there is a single species of solute giving different solid forms in different parts of the curve, the position of the breaks, representing transition points, will be invariant. A further complication is introduced when the solid is precipitated in a macrocrystalline form, as opposed to a microcrystalline or amorphous form; Smithies (1954) describes anomalous breaks in the precipitation curve of lactoglobulin which were the result of crystallization.

In this paper the results of experiments on the solubility of normal adult human carboxyhaemoglobin in ammonium sulphate at pH 6·8 are reported.

MATERIAL AND METHODS OF INVESTIGATION

Haemoglobin specimens were obtained from three normal adult subjects, two male and one female, as follows. About 10 ml. of blood was collected into 0·2 M-citrate buffer, pH 7·4, with Na^+ as the only cation and 200 mg. of glucose/100 ml. The erythrocytes were washed three times in a large volume of 0·85% NaCl. After the final packing of the cells, an equal volume of distilled water was added, and the mixture was frozen twice to lyse all the cells. Carbon monoxide was bubbled through the haemolysate, which was then dialysed for 24 hr. at 4° against several changes of distilled water saturated with CO. The haemolysate was centrifuged at 5000 *g* for ½ hr. to sediment the cell stroma. The clear supernatant was decanted and the concentration of carboxyhaemoglobin measured in a Unicam SP. 600 spectrophotometer at 539 mμ. The aqueous solution of carboxyhaemoglobin was diluted 1 to 20, 30 and 40 (v/v) with M-phosphate buffer, pH 6·8 (prepared by mixing M-NaOH with M-KH_2PO_4). Then 1 ml. samples of the mixtures were pipetted into 6 ml. centrifuge tubes, followed by 4 ml. of $(NH_4)_2SO_4$ solution to give the final concentration required. All solutions had been saturated with CO. The tubes were sealed with silicone-treated rubber bungs, and, after being mixed by inversion, were allowed to stand at room temperature (18±3°) for 24 hr. The tubes were centrifuged at 5000 *g* for 20 min. to sediment the precipitate. The protein concentration in the supernatant was determined by its absorption at 539 mμ.

A constant-solvent phase diagram, of the type devised by Northrop & Kunitz (1930), was also constructed. An appropriate final concentration of $(NH_4)_2SO_4$ was selected as the solvent by inspection of the variable-solvent curve. The protein concentration was varied by carefully pipetting different amounts of the buffered haemoglobin solution used in the variable-solvent test into a series of tubes. The volume was made up to 1 ml. with buffer and 4 ml. of $(NH_4)_2SO_4$ was added, to give the same final concentration of buffer and salts in each tube. After standing at room temperature for 24 hr. the concentration of protein which remained in solution was determined as before.

RESULTS

The variable-solvent salting-out curve of normal adult human carboxyhaemoglobin at pH 6·8 in ammonium sulphate solution is shown in Fig. 1. In the curve three breaks occur, the positions of which are all dependent on the concentration of haemoglobin originally in solution. Similar results were obtained with samples from three subjects. The absorption spectra of the carboxyhaemoglobin remaining in solution and in the redissolved precipitates were indistinguishable.

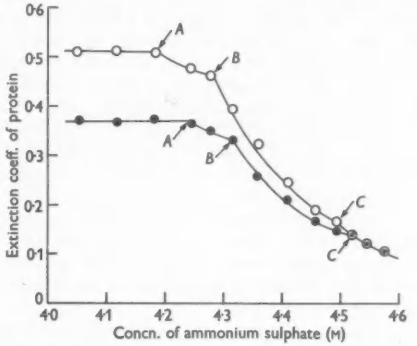


Fig. 1. Precipitation curves of normal adult human carboxyhaemoglobin in aqueous ammonium sulphate, pH 6·8, 18°. Discontinuities in the curves are shown by arrows *A*, *B*, *C*.

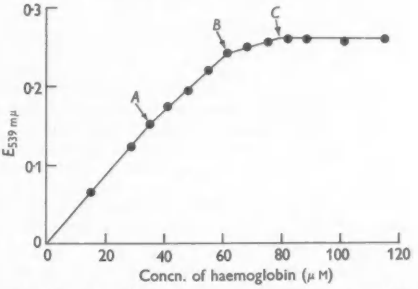


Fig. 2. Variable-solute phase diagram in aqueous 4·36 M-ammonium sulphate, pH 6·8, 18°. Discontinuities in the plot occur at the points indicated by arrows *A*, *B*, *C*.

The constant-solvent phase diagram is shown in Fig. 2. Three breaks are seen, as expected of a three-component system. Precipitation was visible at haemoglobin concentrations above the first break. The precipitate was examined microscopically in some experiments at a magnification of 600 and did not appear to be crystalline.

DISCUSSION

Our results are similar to the ammonium sulphate solubility curves obtained with horse carboxyhaemoglobin by Roche *et al.* (1954). Three breaks in the precipitation curve have been found, the position of all being dependent upon the concentration of protein originally in solution. There was no evidence of crystallization, and it is concluded that normal adult human carboxyhaemoglobin comprises three molecular species.

Kunkel & Wallenius (1955) submitted normal adult human carboxyhaemoglobin to electrophoresis in starch slabs and obtained three components. The eluted components all had the same absorption spectra as the original pigment, and when they were submitted to electrophoresis separately they retained the mobility shown in the first experiment. It is therefore unlikely that these components represent denaturation products, or different forms of a single chemical substance. Morrison & Cook (1955) were also able to obtain three components from normal adult oxyhaemoglobin by chromatography on a column of Amberlite IRC-50 ion-exchange resin. The spectra of the components were found to be normal, from which it was concluded that no significant denaturation had taken place.

The discontinuities in the precipitation curve at high concentrations of phosphate reported by Roche *et al.* (1953*a*, *b*) and attributed by them to the presence of three small foetal haemoglobin components in the haemoglobin of normal adults, were not observed in our experiments. It was not possible in our tests to obtain very high salt concentrations without modifying the conditions, so that we have not excluded the possibility that other breaks occur in this region. However, other results, including accurate measurements of the rate of denaturation of haemoglobin in alkali (Beaven, Ellis & White, 1956), suggest that the proportion of foetal haemoglobin in the blood of normal adults is less than 1 % of the total, if any is present at all.

SUMMARY

The results of variable-solvent and variable-solute tests indicate that normal adult human carboxyhaemoglobin contains three distinct molecular species, thus confirming the heterogeneity observed by Kunkel & Wallenius by electrophoresis and by Morrison & Cook by chromatography on ion-exchange resin columns.

The authors are indebted to Drs A. G. Ogston and R. Cecil for their criticism of the manuscript.

REFERENCES

Beaven, G. H., Ellis, M. & White, J. C. (1956). *Nature, Lond.*, **178**, 857.
Cohn, E. J. (1925). *Physiol. Rev.* **5**, 349.
Huisman, T. H. J. & Prins, H. K. (1955). *J. Lab. clin. Med.* **46**, 255.
Itano, H. A. (1956). *Annu. Rev. Biochem.* **25**, 331.
Kunkel, H. G. & Wallenius, G. (1955). *Science*, **122**, 288.
Morrison, M. & Cook, J. L. (1955). *Science*, **122**, 920.
Northrop, J. H. & Kunitz, M. (1930). *J. gen. Physiol.* **13**, 781.
Ogston, A. G. & Tombs, M. P. (1956). *Nature, Lond.*, **178**, 200.
Roche, J. & Derrien, Y. (1953). *Sang*, **24**, 97.
Roche, J., Derrien, Y., Reynaud, T., Laurent, G. & Roques, M. (1954). *Bull. Soc. Chim. biol., Paris*, **35**, 51.
Roche, J., Derrien, Y. & Roques, M. (1953*a*). *C.R. Soc. Biol., Paris*, **146**, 689.
Roche, J., Derrien, Y. & Roques, M. (1953*b*). *Bull. Soc. Chim. biol., Paris*, **35**, 933.
Smithies, O. (1954). *Biochem. J.* **58**, 31.

Determination of Creatine in Biological Fluids

By D. R. ANDERSON, C. M. WILLIAMS, G. M. KRISE and R. M. DOWBEN*
The Radiobiological Laboratory, Balcones Research Center, Austin, Texas, and the Department of Radiobiology, School of Aviation Medicine, Randolph Field, Texas

(*Received* 25 *January* 1957)

The colour reaction between diacetyl and creatine in the presence of α-naphthol can be used for the determination of creatine (Barritt, 1936). A number of inhibitory substances occur in urine, plasma and tissue extracts which interfere with the formation of the coloured product (Eggleton, Elsden & Gough, 1943). It was shown by Ennor & Stocken (1948) that sulphydryl compounds could produce marked inhibition of the diacetyl reaction and that the interference could be prevented by the addition of *p*-chloromercuribenzoic acid. On applying their method to urine, Ennor & Stocken (1953) found that

* Present address: Department of Medicine, Northwestern University, Chicago 11, Illinois, U.S.A.

other unknown urinary constituents also hindered the diacetyl reaction. They reported that the interference could be overcome in part by increasing the concentration of α-naphthol.

In determinations of creatine in rat urine according to the directions of Ennor & Stocken (1953), adequate recovery of added creatine was not obtained. The addition of *p*-chloromercuribenzoic acid did not improve the results. An attempt then was made to separate the inhibitors of the diacetyl reaction from creatine by means of ion-exchange chromatography. It was found that creatine is not readily absorbed on either strong anion or weak cation exchangers. Provided the concentration of other cations is not high, it is absorbed, however, on a strong cation-exchange resin, from which it may be then eluted with sodium acetate. No interference in the diacetyl reaction was observed after the separation of the creatine by chromatography.

EXPERIMENTAL

Reagents

Creatine. Reagent-grade creatine (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was recrystallized twice from hot water and dried over $CaCl_2$. The water of crystallization was driven off by heating at 120°. Paper chromatograms on Whatman no. 1 paper with the solvents in Table 3 gave a single spot with the diacetyl test at the R_F corresponding to creatine and no spot with the Jaffe test at the R_F corresponding to creatinine (Found: N, 31·9. Calc. for $C_4H_9O_2N_3$: N, 32·0%).

Stock alkali. Sodium hydroxide (60 g.) and Na_2CO_3 (160 g.) in 1 l. of water was used.

Diacetyl. Diacetyl (Nutritional Biochemicals Corp.) was purified by distillation in 100 ml. batches; all but the first and last 10 ml. portions of the fraction distilling at 88° was used. A 1% stock solution in water was diluted 1:20 immediately before use.

α-Naphthol. The commercial reagent was recrystallized from 0·01 N-HCl. The purified α-naphthol can be stored in a desiccator in the dark for several months. A 1·6 or 3·2% solution in stock alkali is prepared immediately before use.

Ion-exchange resins. Amberlite IRA-401 (strong anion exchanger), Amberlite IRC-50 (weak cation exchanger), and Amberlite IR-120 (strong cation exchanger) were obtained from Rohm & Haas Co. (Philadelphia, Pa., U.S.A.) as porous beads (30–60 mesh). The anion exchanger was converted into the hydroxide cycle by immersion in 0·1 N-NaOH. The cation exchangers were converted into the hydrogen cycle with 0·1 N-HCl. All resins were then washed with water until the effluent was neutral.

Cellulose. Whatman powdered cellulose was used.

Solvents. Reagent-grade solvents [Eastman Kodak Co. (Rochester, N.Y., U.S.A.) or Matheson Co. Inc. (Norwood, Ohio, U.S.A.)] were used without further purification. 2-Picoline obtained from two different sources gave somewhat different R_F values; for this reason, when 2-picoline is used in a developer, the R_F values should be determined for each batch.

Creatinine. A stock solution containing 1 mg./ml. in 0·1 N-HCl was obtained from Hartman-Leddon Co. Inc. (Philadelphia, Pa., U.S.A.).

Guanidinoacetic acid and arginine. Guanidinoacetic acid (Found: N, 35·9. Calc. for $C_3H_7O_2N_3$: N, 35·9%) and arginine (Found: N, 32·1. Calc. for $C_6H_{14}O_2N_4$: N, 32·2%) (Nutritional Biochemicals Corp.) gave single spots on paper chromatograms (Table 1).

p-Chloromercuribenzoic acid. Reagent-grade *p*-chloromercuribenzoic acid (142 mg.) (K & K Laboratories, Long Island City, New York, U.S.A.) was dissolved in the minimum amount of N-NaOH and diluted to 100 ml. with water.

Determination of creatine in urine

A sample (approx. 3 ml.) of urine containing not more than 3–4 mg. of creatinine is diluted 1:5 and passed through four consecutive columns (7 mm. diameter) containing 10–15 mm. of cellulose, 200 mm. of Amberlite IRA-401, 200 mm. of Amberlite IRC-50, and 200 mm. of Amberlite IR-120 respectively. The columns are mounted at successively lower levels in a rack. The lower end of each tube is plugged with a plastic adapter and an 18-gauge hypodermic needle which is fitted with a polythene tube leading to the next column. The last tube is fitted with an adapter and adjustable valve to regulate the outflow. The outflow is adjusted to equal the inflow (about 15–20 drops/min.). The other columns are arranged so that the hydrostatic pressure and siphoning action give continuous flow of added liquid without any column running dry. After the urine has been added, the column train is washed with about 200 ml. of water. Creatine is eluted from the Amberlite IR-120 column with sufficient N-sodium acetate to make 50 ml. of eluate.

A 7 ml. portion (containing 10–40 μg. of creatine) is mixed with 2 ml. of 1·6% α-naphthol and 1 ml. of 0·05% diacetyl is added with gentle swirling. The optical density is measured at 525 mμ after 20 min. but before 30 min. The coloured product is stable until 30 min. (Ennor & Stocken, 1953) and then diminishes somewhat in intensity during the next hour.

The resin columns may be regenerated and used for eight to ten runs before replacement with new resins is necessary.

Determination of creatine in plasma and tissue extracts

Plasma or tissue extract (3–6 ml.) is added to a 50 ml. flask containing 3 ml. of Amberlite IRA-401 and 3 ml. of Amberlite IRC-50, and the volume is brought to 25 ml. with water. The flask is shaken for 1 hr. at room temp. The contents are filtered, the residue is washed with 200–300 ml. of water. Filtrate and washings are run through a column (7 mm. × 70 mm.) of Amberlite IR-120. Creatine is eluted from the column with N-sodium acetate to volume in a 25 ml. volumetric flask. In plasma, a small amount of protein, which interferes with the colorimetric determination, is eluted with the creatine. This residual protein is removed by adding 1 ml. of stock alkali to 7 ml. of eluate. The precipitate is removed by centrifuging after 15 min. To the supernatant, 1 ml. of 3·2% α-naphthol and 1 ml. of diacetyl solution are added. The optical density is measured at 525 mμ at 20 min.

Table 1. *R_F values for creatine, creatinine, arginine, and guanidinoacetic acid*

The ascending method with Whatman no. 1 paper was used. Time of run: 18 hr. at 25°.

Solvent	Creatinine	Creatine	Arginine	Guanidinoacetic acid
2-Picoline–water (9:1, v/v)	0·31	0·03	0·01	0·03
Acetone–30% acetic acid (6:4, v/v)	0·74	0·59	0·43	0·59
2-Picoline–aq. NH_3 soln. (sp.gr. 0·88)–water (6:2:1, by vol.)	0·62	0·32	0·20	0·34
Pyridine–aq. NH_3 soln. (sp.gr. 0·88)–water (6:2:1, by vol.)	0·84	0·61	0·47	0·65
Dimethylformamide–aq. NH_3 soln. (sp.gr. 0·88)–water (6:2:1, by vol.)	0·78	0·43	0·31	0·41

Paper-chromatographic separation of creatine and creatinine

Milligram samples of pure creatine and creatinine may be separated on paper, for example, for assay of specific radioactivity. Urine (100–200 ml.) is first desalted by running it through successive columns of cellulose, Amberlite IRA-401 and Amberlite IRC-50. The volume of the eluate is reduced to 10 ml. with a stream of air. The concentrated solution is stripped on 18 in. × 22 in. sheets of Whatman no. 1 paper mounted on a drum which rotates (Yanofsky, Wasserman & Bonner, 1950). The chromatograms are developed by ascending chromatography with the solvents listed in Table 1 for 12–18 hr. at 25°. Test strips are cut from the edges of the large sheet.

Creatine is detected by dipping the strips into each of the following solutions in succession: (1) methanol, (2) 6% (w/v) NaOH in methanol, (3) 400 mg. of α-naphthol and 3 g. of NaOH in 25 ml. of methanol, and (4) diacetyl solution (stock diacetyl solution diluted 1:10 with methanol). Creatine gives a red band at room temp. after 5–10 min. This test gives the most intense colour with creatine, but arginine and guanidinoacetic acid give positive, albeit less strong, reactions.

For the detection of creatinine the strip is dipped into a mixture of 15 ml. of 1·2% picric acid in methanol and 5 ml. of 6% NaOH in methanol, blotted lightly and allowed to dry. After a few min. at room temp. an orange band characteristic of creatinine appears against the yellow background.

Arginine and guanidinoacetic acid were located by means of an alkaline sodium nitroprusside–potassium ferricyanide spray (Berry, Sutton, Cain & Berry, 1951). Arginine may be identified in the presence of creatine and guanidinoacetic acid by use of ninhydrin in butanol.

RESULTS

In the methods described, the cellulose is used to remove colloidal and particulate matter. In the method for urine the electrolytes are largely removed by the first two resin columns. The Amberlite IRC-50 column absorbs 95–100% of the arginine present in amount up to 1 mg. and 96–100% of the creatinine present in amounts up to 4·5 mg. (Table 2). Guanidinoacetic acid is absorbed by the Amberlite IR-120 column and eluted by the sodium acetate along with the creatine. The observed value of creatine with the diacetyl colour test is about 11–13% high when guanidinoacetic acid and creatine are present in equal quantity in urine. Recoveries of known amounts of creatine added to several urine specimens in concentrations up to 1 mg./ml. ranged between 95·0 and 98·5%.

Table 2. *Removal of creatinine from urine by IRC-50 ion-exchange resin*

Urine was passed through a 200 mm. column (7 mm. diam.) of Amberlite IRA-401 and then through a 200 mm. column of Amberlite IRC-50. Creatinine in the effluent was determined by the method of Owen, Iggo, Scandrett & Stewart (1954).

Urine (ml.)	Creatinine in original sample (μg.)	Creatinine in effluent from IRC-50 column (μg.)	Amount absorbed on IRC-50 column (%)
1	1210	45	96·5
1	1400	0	100·0
2	2200	30	98·5
3	4080	105	97·5
3	4400	137	97·0

Attempts were made to obtain higher readings from the eluted samples by counteracting possible interference from sulphydryl compounds with *p*-chloromercuribenzoic acid. Values for creatine with and without *p*-chloromercuribenzoic acid were found to be the same.

When it was necessary to wait longer than 1–2 hr. for analysis, samples were preserved by freezing. At −10°, no change in creatine concentration occurs over a period of several weeks. The mean value of creatine excretion for eight normal men was 48·4 ± 26·2 mg./24 hr.

Several other methods for the deproteinization of plasma and tissue extracts were found unsatisfactory. Protein-free filtrates obtained with use of trichloroacetic acid were not suitable because α-naphthol reacts with trichloroacetic acid in light to form a blue substance (Eggleton *et al.* 1943). Methods which employ strong acids cause the conversion of creatine into creatinine, and strong bases cause the conversion of creatinine into creatine. Paper chromatography of plasma before

Table 3. *Recovery of creatine added to 3 ml. of rat plasma*

Creatine (added) (μg.)	Total creatine (calc.) (μg.)	Creatine (found) (μg.)	Recovery (%)
0	—	52·8	—
20	72·8	72·0	96
20	72·8	73·4	103
40	92·8	93·8	102
40	92·8	92·4	99
40*	40·0	38·6	97
40*	40·0	41·7	104

* Added to 3 ml. of saline solution.

deproteinization was not satisfactory because creatine was trapped in the large amount of protein. Creatine and creatinine migrated toward the cathode in a multiple-cell electrodialysis unit, but quantitative separation from the plasma compartment was not complete after 8 hr. No interconversion of creatine and creatinine occurs on the resins.

p-Chloromercuribenzoic acid was added to duplicate tubes of plasma samples without effect on the value of creatine. It was observed, however, that *p*-chloromercuribenzoic acid increased the observed value of creatine in red-cell haemolysates, and the inhibition of the diacetyl reaction in whole-blood filtrates (Ennor & Stocken, 1948) may be due to substances present in the red cells.

By the described method, recoveries ranging from 96·1 to 104·0 % were obtained from addition of creatine to plasma (Table 3). In eight healthy adult males, the plasma creatine was $2·9 \pm 1·5$ mg./ml.

DISCUSSION

Before the Barritt (1936) modification of the Voges–Proskauer diacetyl reaction, creatine was measured by the Jaffe reaction after conversion into creatinine by heating with acid. The Jaffe reaction itself is non-specific (Hunter, 1928), and considerable controversy has existed about the absolute value of creatinine so measured. Two methods for the accurate determination of creatinine have come into general use: the enzymic method of Miller & Dubos (1937) and the adsorption of creatinine on Lloyd's reagent and subsequent determination by the Jaffe reaction (Borsook, 1935). Owen *et al.* (1954) compared the methods critically and concluded that the most convenient compromise suitable for routine use was the latter method. The measurement of creatine by the difference of creatinine values before and after heating with acid is subject to gross errors. Heat and acid may give rise to other Jaffe-positive chromogens; guanidinoacetic acid, for example, is converted into the Jaffe-positive chromogen glycocyamidine by heat and acid (Hunter, 1928). When a small amount of creatine is present with large amounts of creatinine, as in urine, there are the difficulties of accurately measuring a small difference. The use of the Barritt (1936) modification of the diacetyl reaction for the routine analysis of biological fluids has been limited by the presence of inhibitors which interfere with the reaction (Eggleton *et al.* 1943; Ennor & Stocken, 1948, 1953). We have overcome this difficulty by the prior separation of creatine by ion-exchange chromatography.

Arginine, guanidine and guanidinoacetic acid react with diacetyl to give a colour about one-ninth of the intensity of an equivalent amount of creatine. Creatinine and creatine phosphate do not react under the conditions described (Eggleton *et al.* 1943). Of the guanidinium compounds which react in the diacetyl test, only arginine and guanidinoacetic acid are likely to be present in urine and plasma in amounts large enough to cause interference (Van Pilsum, Martin, Kito & Hess, 1956). Arginine is completely removed and guanidinoacetic acid is removed to a considerable extent by the ion-exchange resins. Thus the creatine values obtained by this method are high by about 10 %. For very accurate determinations, the amount of guanidinoacetic acid present may be determined by the Sakaguchi reaction and the creatine values corrected. In creatinuria, the error produced by the presence of guanidinoacetic acid is negligible.

Creatinine is slowly converted into creatine in the alkaline condition under which the diacetyl-colour reaction is performed. Prior removal of the creatinine by ion-exchange chromatography eliminates the slow increase in creatine and in colour with time.

SUMMARY

1. A method is described for the determination of creatine in biological fluids by the colour reaction with diacetyl in the presence of α-naphthol. The creatine is partly separated before the colour reaction by ion-exchange chromatography.

2. Ion-exchange chromatography removes inhibitors of the diacetyl reaction, arginine (which gives a coloured product with diacetyl) and creatinine.

3. The separation and detection of creatine, creatinine and related compounds by paper chromatography is described.

REFERENCES

Barritt, M. M. (1936). *J. Path. Bact.* **42**, 441.

Berry, H. K., Sutton, H. E., Cain, L. & Berry, J. S. (1951). *Biochemical Institute Studies*, vol. 4. Austin, Texas: University of Texas Press.

Borsook, H. (1935). *J. biol. Chem.* **110**, 481.
Eggleton, P., Elsden, S. R. & Gough, N. (1943). *Biochem. J.* **37**, 526.
Ennor, A. H. & Stocken, L. A. (1948). *Biochem. J.* **42**, 557.
Ennor, A. H. & Stocken, L. A. (1953). *Biochem. J.* **55**, 310.
Hunter, A. (1928). *Creatine and Creatinine.* London: Longmans Green and Co.
Miller, B. F. & Dubos, R. (1937). *J. biol. Chem.* **121**, 457.
Owen, J. A., Iggo, B., Scandrett, F. J. & Stewart, C. P. (1954). *Biochem. J.* **58**, 426.
Van Pilsum, J. F., Martin, R. P., Kito, E. & Hess, J. (1956). *J. biol. Chem.* **222**, 225.
Yanofsky, C., Wasserman, E. & Bonner, D. M. (1950). *Science*, **111**, 61.

Hormones and Liver Cytoplasm

4. RIBONUCLEOTIDES, RIBONUCLEIC ACID SYNTHESIS AND PROTEIN SYNTHESIS AFTER ADRENALECTOMY*

By E. REID and BARBARA M. STEVENS†
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3

(*Received 5 March* 1957)

After adrenalectomy, the supernatant fraction obtained from rat liver by differential centrifuging shows increases in the amount of ribonucleic acid (RNA) (Reid, 1955) and in the incorporation of injected [6-^{14}C]orotic acid into the RNA (Reid, O'Neal, Stevens & Burnop, 1956). On the other hand, the microsomal fraction shows a decreased incorporation of orotic acid. This decrease is in accordance with the postulated role of microsomal RNA in protein synthesis, the rate of which as judged by the incorporation of injected DL-[1-^{14}C]-leucine appears to be slightly diminished after adrenalectomy (Reid *et al.* 1956). No attempt was made in these studies to ascertain whether adrenalectomy affected the precursor 'pool' or the time course of incorporation. It was not therefore conclusively proved that the increase in the amount of RNA in the supernatant fraction is due to increased synthesis as distinct from decreased catabolism or that this RNA has some function unconnected with protein synthesis.

A closer study has now been made in an endeavour to clarify not only these aspects but also the origin, the fate and the possible metabolic diversity of the RNA of the supernatant fraction.

Liver ribonucleotides have also been investigated, to ascertain whether adrenalectomy affects the ribonucleotide pool and, in particular, the pool of the uridine phosphates into which injected orotic acid is rapidly converted (Hurlbert & Potter, 1952, 1954; Schmitz, Hurlbert & Potter, 1954).

Another aspect which has now been studied, as briefly reported elsewhere (Reid & Stevens, 1956), is the sequence after adrenalectomy of the various changes in the metabolism of RNA and of protein.

* Part 3: Stevens & Reid (1956).

† Royal Marsden Hospital Gordon Jacobs Fellow.

EXPERIMENTAL

Experimental procedures are described in detail only where these differ from those employed in Part 1 (Reid *et al.* 1956). As previously, the animals were male rats, wt. about 250 g., which had been fed on a protein-rich diet in restricted amount and which were fasted overnight before autopsy. Adrenalectomized rats were maintained with saline.

Chromatography of ribonucleotides

Each experiment was performed with pooled liver samples (total 10–11 g.) from two rats. In the later experiments, as indicated in Table 1, the rats were killed by decapitation, and the liver was ground to a powder in the frozen state (liquid nitrogen) before extraction with perchloric acid solution, as described by Hurlbert & Potter (1954) and Hurlbert, Schmitz, Brumm & Potter (1954) but without perfusion of the liver.

Analysis of the neutralized extracts was performed by anion-exchange chromatography on Dowex 1 (formate) columns, by a gradient-elution procedure with three changes of solvent as described by Hurlbert *et al.* (1954). The pH of the solvents was not adjusted. The mixing flask (containing water initially) was of 500 ml. volume, and the resin column 15 cm. × 0·72 cm.^{2} in size; with a longer column (20 cm.) the resolution was little improved. The effluent was collected in 5 ml. portions (about 300 in each run) which were examined spectrophotometrically at 260 mμ and, in some instances, at 275 mμ. In general, each run was carried out for 3 days without interruption of the flow, the elution pattern being distorted after any interruption. In experiments with radioactive material, performed in a distant laboratory with consequent difficulty in supervision, the runs were shortened by using only two solvents (4N-formic acid followed by 4N-formic acid containing M-ammonium formate), with resulting loss of resolution in the later stages (Expt. 2 of Table 1).

Some of the fractions thus obtained were rechromatographed after removal of water and formic acid by freeze-drying; if the fraction also contained ammonium formate, this was removed by the cautious use of an infrared lamp in

the final stage of drying as in the experiments of Hurlbert *et al.* (1954) with the aid of a mercury-diffusion pump to achieve a higher vacuum. Rechromatography of heterogeneous fractions was carried out by gradient elution with ammonium formate solutions, as described by Hurlbert *et al.* (1954); here the mixing flask was of 250 ml. vol., and the resin column 10 cm × 0·72 cm.² in size. With careful drying, about 80% of the ultraviolet light-absorbing material eluted in the initial chromatograms could be recovered after the rechromatography.

Identification of the nucleotides in the various peaks was based on the data of Hurlbert *et al.* (1954), and was confirmed by chromatographic data obtained with authentic nucleotides (Sigma Chemical Co., St Louis, Mo., U.S.A.) and by examination of the peaks with respect to the ratio of the extinctions at 275 and 260 mμ. The chromatograms of the crude tissue extracts showed certain minor qualitative divergences from the data of Hurlbert *et al.* Triphosphopyridine nucleotide (TPN) ran ahead of, rather than with, guanosine 5′-phosphate (GMP). Inosine 5′-phosphate (IMP) and uridine 5′-phosphate (UMP) were not separable without rechromatography (even with the authentic nucleotides in place of the tissue extract), but uridine 5′-pyrophosphate glucose (UDPglucose) was obtained free of guanosine 5′-pyrophosphate (GDP); in these respects the present findings accord with those of Schmitz, Potter, Hurlbert & White (1954). Guanosine triphosphate (GTP) merged with uridine triphosphate (UTP), and UDP and its glucuronic acid derivative merged with adenosine triphosphate (ATP).

Diphosphopyridine nucleotide (DPN) and its reduced form (DPNH) were estimated in freshly excised liver samples by the use of alcohol dehydrogenase according to Spirtes & Eichel (1954). The rats were killed by decapitation, the values tending to be lower if Nembutal (pentobarbital sodium) was used.

Isotopic studies

The number of rats used in each experiment is stated in the legends to the figures; in general each point is derived from data for two to four rats. Adrenalectomized rats were always studied simultaneously with control rats (intact or sham-operated) because of possible day-to-day variations in environment or technique.

In some experiments the interval between adrenalectomy and injection of isotope was deliberately varied. Since these experiments, which are described below, showed that the effects of adrenalectomy were quantitatively dependent on the length of this interval, it was necessary to standardize this where other variables were to be investigated. The length of the interval was 12–15 days unless otherwise stated.

Isolation of fractions. As in the previous study (Reid *et al.* 1956), homogenates of the liver samples removed at autopsy were centrifuged to give nuclear and cytoplasmic fractions. Further centrifuging of the cytoplasmic fraction gave a mitochondrial fraction (with 'fluffy layer'), which was discarded, a microsomal fraction and a supernatant fraction, i.e. the material unsedimented after 90 min. at 14 500 *g*. In some experiments the latter fraction was centrifuged for 60 min. at 145 000 *g* to sediment an ultracentrifugal fraction.

In some instances supernatant fractions were further fractionated by procedures other than ultracentrifuging; here each point on the figures was obtained not with several supernatant fractions, but usually with a pooled fraction from a pair of rats which had been similarly treated. Ammonium sulphate precipitation was carried out by addition of saturated $(NH_4)_2SO_4$ solution at 0–3°, with high-speed centrifuging (10 min. at 14 500 *g*) so as to give a clear supernatant and a compact sediment, which was not washed. Precipitation with Ca^{2+} ions (Schneider, 1946), by addition of $CaCl_2$ solution to a final concentration of 0·1% $CaCl_2$, was carried out at room temperature; high-speed centrifuging was not necessary.

As previously (Reid *et al.* 1956), RNA was estimated from the extinction at 260 mμ of acidified alkaline digests. The validity of this procedure, as applied to subfractions separated from the supernatant fraction, was confirmed by analyses for RNA phosphate.

In preparing a powder for measurement of radioactivity, the initial removal of acid-soluble and lipid constituents from tissue samples was usually performed with perchloric acid solution followed by treatment at room temperature with ethanol–ether–chloroform (2:2:1, by vol.), as in the experiments of Littlefield, Keller, Gross & Zamecnik (1955). The use of hot ethanolic solvents, as in the previous study (Reid *et al.* 1956), was less convenient and, moreover, might have resulted in some loss of protein (Korner & Debro, 1956). Trichloroacetic acid solution was used in place of perchloric acid solution when it was intended to measure the acid-soluble radioactivity (see below) or when nuclear preparations were being treated; the latter tended to become fibrous if treated with perchloric acid.

Plating of liquid samples. Liquid samples were dried and counted at slightly less than infinite thickness, with a standard amount of carrier sufficient to minimize differences due to any variations in the amount of tissue solid. Hurlbert & Potter (1954) used ammonium trichloroacetate for this purpose; but with this alone, erratic results due to uneven drying have now been encountered, even with nickel planchets in place of polyethylene ones. With $BaSO_4$ as an additional constituent, as described below, duplicates seldom differed by more than 5%.

With cytoplasmic fractions, the tissue extracts, containing 7% (w/v) trichloroacetic acid, were prepared for counting by pipetting 0·1 ml. into the well of a polyethylene planchet (2 cm.²), followed by 0·13 ml. of 0·5 N-$Ba(OH)_2$ solution and 0·013 ml. of 2 N-H_2SO_4 solution. Finally an excess of aq. NH_3 soln. (sp.gr. 0·88) was added, with stirring so as to disperse the precipitate of $BaSO_4$. The contents of the planchet were carefully taken to dryness by exposure for about 3 hr. to an infrared lamp.

With fractions obtained by anion-exchange chromatography in the formate system, ammonium formate was used as carrier in place of trichloroacetic acid. To 0·15 ml. of the effluent, sufficient 90% (w/w) formic acid solution was added to bring the total amount of formic acid present to 1·15 m-moles (e.g. 0·05 ml. added if none already present; 0·04 ml. was added to effluents containing UMP). After addition of 0·05 ml. of 0·5 N-$Ba(OH)_2$ solution and 0·02 ml. of 2 N-H_2SO_4 solution, excess of aq. NH_3 soln. was added with stirring. Even with very careful drying, so as to minimize sublimation of the ammonium formate, the results were less reproducible than with ammonium trichloroacetate as carrier, and it was essential to run several replicates.

Injection of isotope. Injections were given intraperitoneally unless otherwise stated. In general, the dosage chosen was the lowest amount compatible with accuracy in the

final counting, this amount being in the range 2–8 μc, with orotic acid as precursor and with an interval exceeding 1 hr. between injection and autopsy. With a long interval (20 or 30 hr.), a comparison of different doses within the range 0·5–8 μc indicated that the percentage recovery in the RNA of microsomal and supernatant fractions decreased somewhat as the dose was increased. However, the decrease was not marked in relation to day-to-day variations in percentage recovery, and moreover the dosage within each group was the same for adrenalectomized rats as for the controls. Accordingly, no attempt has been made to correct for differences in dosage from one experiment to another. In experiments with an interval of only 40 min. or 1 hr., high doses of orotic acid (up to 40 μc, i.e. 6·3 mg. of orotic acid) were employed so as to ensure measurable labelling of microsomal RNA and of uridine compounds in the acid-soluble fraction; in some of these experiments the dose may have approached 'saturation level'.

Since the incorporation of orotic acid at short time intervals was low but accurately measurable, logarithmic axes have been adopted in plotting the results. These are considered in terms of percentage recovery of injected isotope except where their assessment in terms of specific activity is advantageous; the amounts and specific activities of RNA pyrimidines were not actually determined. It appeared appropriate in experiments requiring a standardized interval to adopt an interval of 135 min. after injection of orotic acid, as previously (Reid *et al.* 1956).

In experiments with leucine the standardized interval was 90 min. Data obtained at shorter time intervals (15 and 40 min.) are also given below: here L-leucine (generally labelled with ^{14}C; Radiochemical Centre, Amersham) was employed in place of DL-[1-^{14}C]leucine, usually at a dosage of 0·063 mg. equivalent to 6 μc.

Injection of unlabelled orotic acid. Intact rats were given massive doses of orotic acid (Genatosan Ltd., Loughborough, Leics.) to ascertain the effect upon liver RNA levels. The compound was given as an almost saturated solution in water at 37° and at pH 8·5 (3 mg./ml.); 5 ml. was injected intraperitoneally, followed by four similar injections at hourly intervals, and the rats were killed 2 hr. after the last injection. Control rats were given water containing a trace of sodium bicarbonate.

RESULTS

Liver ribonucleotides

Effects of improvements in technique. Since Hurlbert & Potter (1954) and Hurlbert *et al.* (1954) do not emphasize that replication of their results depends on scrupulous adherence to their techniques, as became evident during the present experiments, the data for control rats (Tables 1 and 2) may usefully be considered from this aspect before the effects of adrenalectomy are discussed. With practice in techniques it became possible to treat the tissue more expeditiously before extraction and, in particular (Expts. 4*c et seq.*), to grind the frozen tissue to a powder before extraction. As is evident from the data for control rats, in the earlier experiments there were post-mortem changes, namely decreases in the levels of triphosphates (ATP, UTP, GTP) and of certain diphosphates and their derivatives [adenosine diphosphate (ADP), UDPglucose, UDPglucuronic acid and UDP] together with increases in the levels of certain monophosphates [adenosine monophosphate (AMP) and GMP] and possibly of TPN. On the other hand, the levels of DPN, of UMP and of UDPacetylglucosamine were remarkably constant throughout. In general, the patterns obtained in the final experiments quantitatively resembled those of Hurlbert *et al.* (1954) and of Schmitz *et al.* (1954).

At the outset of the study it was hoped that nucleotide levels could be determined in individual fractions isolated from sucrose homogenates. However, it soon became evident that considerable breakdown of nucleotides occurs in the course of separation of nuclei and mitochondria. This conclusion is, of course, in accord with the later findings described above.

Effects of adrenalectomy. There was a striking decrease in the amounts of uridine nucleotides 2–3 weeks after adrenalectomy (Tables 1 and 2). Rechromatography of fraction (5) of Table 1 showed no change in IMP but a highly significant decrease in UMP ($P < 0{\cdot}5\%$; Table 2). The results for the labile compound UDPglucose (and possibly those for ADP) suggest that adrenalectomy may decrease its level *in vivo* but minimize its breakdown *post mortem*. The results for UDP, UDPglucuronic acid and UTP (Table 2) indicate marked decreases after adrenalectomy; here there is the difficulty that ribonucleoside diphosphates and their derivatives may undergo some breakdown after the initial isolation (Hurlbert *et al.* 1954). However, the total recovery of uridine nucleotides from fraction (11) showed a significant decrease ($P < 5\%$) after adrenalectomy (Table 2). In general, the decreases in the various uridine nucleotides of the liver appeared to exceed 50 % except with UDPacetylglucosamine (29 % decrease) and UDPglucose.

The results for UMP and UDPacetylglucosamine in control rats are very consistent, and the single experiment with adrenalectomized rats 3 days after operation permits the conclusion that these compounds are undiminished after only 3 days; it also appears that the amount of UDPglucose is undiminished or even elevated.

The marked decrease in the uridine nucleotide pool is not reflected in the results for adenosine and guanosine nucleotides, with the possible exception of the triphosphates. Indeed, the results for AMP, the amount of which was affected by changes in technique, showed a small but significant increase (7 %; $P < 5\%$) when comparison was made between fractions from adrenalectomized rats and fractions from controls studied under similar conditions. No conclusions could be drawn as to cytidine nucleotides because of analytical difficulties.

Table 1. *Chromatography of ribonucleotides in perchloric acid extracts of rat liver*

Control and adrenalectomized rats were studied alternately as indicated by the Expt. nos. In series 1 the rats were killed with Nembutal instead of by decapitation. In series 1–3 the liver was macerated while in a semi-thawed state before extraction, whereas in series 4 and 5 the liver was ground to a powder while frozen (liquid nitrogen), the time between decapitation and freezing of the liver being particularly short (1 min.) in series 5. In view of these improvements in technique, the effects of which are discussed in the text, each run with adrenalectomized rats is, in general, comparable only with the corresponding control run. All quantities refer to an amount of liver corresponding to 100 g. body wt., and are expressed in arbitrary units, namely the calculated optical density (at 260 mμ with water as blank and 1 cm. light path) for the material in the fraction if present in only 1 ml. volume. (Similar units are conventionally used to express the molar extinction coefficients of pure compounds.) Abbreviations used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; MP, monophosphate; DP, diphosphate; TP, triphosphate; A, adenosine; G, guanosine; I, inosine; C, cytidine; U, uridine. (Thus UDP denotes uridine 5′-pyrophosphate.) These abbreviations, and those for unidentified compounds ('C-3', 'J-2', etc.), are identical with those of Hurlbert *et al.* (1954); 'post-ADP' is a peak immediately following ADP, which was not labelled by these authors. For each fraction, minor constituents are italicized and trace constituents are ignored.

Fraction no. ...	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)*	(12)*	(13)
Expt. no.	DPN, *CMP*	AMP	TPN	GMP	UMP, 'C-3', IMP, CDP, '*C-4*'	ADP	'Post-ADP'	UDP-acetylglucosamine	UDP-glucose	GDP, 'G-2', 'G-3', '*G-4*'	UDP-glucuronic acid, UDP, *CTP*	ATP, '*J-3*', '*J-2*'	UTP, GTP, '*K-1*'
Control rats													
1*c*	24·4	62·7	6·5	12·0	31·8	22·0	9·6	10·2	7·8	18·4	21·8		7·9
2*c*	23·9	66·8	4·5	11·7	32·3	25·4	—	14·1		15·7	16·1		—
3*c*	28·0	73·9	6·4	13·2	35·0	34·5	8·3	11·6	12·9	17·7	8·7	12·2	3·7
4*c*	23·0	49·5	4·8	8·1	36·8	60·4	9·7	13·5	19·8	22·2	23·6	22·8	14·6
5*c*	22·5	43·0	3·2	5·0	20·6	51·1	6·0	11·0	*ca.* 17	—	35·8	23·7	12·2
Adrenalectomized rats (2–3 weeks after operation)													
1*a*	—	66·6	7·1	11·1	25·4	34·9	14·1	9·3	13·5	19·9	8·1	11·8	7·3
2*a*	27·8	68·0	6·1	10·6	40·5	30·4	—	29·7		11·5	31·0		—
3*a*	33·4	76·8	7·7	13·3	27·4	36·0	10·5	8·9	14·8	—	—	—	—
4*a*	21·4	57·0	2·8	8·1	22·4	—	—	—	—	—	—	—	—
4*a*′	25·0	56·8	3·0	7·4	19·5	42·1	4·9	7·0	12·5	19·6	15·3	10·5	10·3
5*a*	25·2	45·4	4·2	6·7	25·2	48·8	7·6	8·0	14·7	23·2	36·8		9·9
5*a*″	—	—	—	—	—	—	—	7·7	12·7	—	17·5	14·1	8·1
Adrenalectomized rats (3 days after operation)													
5*a*′	—	—	—	—	28·4	—	—	13·6	34·0	—	—	—	—

* These fractions were never well separated, and the values given in some instances for the two fractions separately may not be individually accurate.

Diphosphopyridine and reduced diphosphopyridine nucleotides. Separate analyses for these compounds were performed with five pairs of rats, each pair consisting of an intact rat and a rat which had been adrenalectomized 2–3 weeks previously. The mean values for DPN and DPNH in the controls were 1·06 and 0·46 mg./100 g. body wt. respectively (1 mg. of DPN is equivalent to 27·7 'units' as defined in Table 1). The mean changes (with standard error) after adrenalectomy were $+0·04 \pm 0·142$ and $-0·05 \pm 0·072$ respectively; these changes are insignificant.

Incorporation of orotic acid in relation to time after injection of isotope

In presenting their data for the time course of labelling of the various RNA fractions, Hurlbert & Potter (1954) remark: 'The data do not offer an accurate kinetic record because too few animals were used to offset the large variations in utilization of the dose in these experiments. The reason for these variations has not been determined....' The more extensive data now obtained show not only quite consistent changes with time in spite of day-to-day variations, but also good agreement with the data given by Hurlbert & Potter (1952, 1954) for the labelling of microsomal, supernatant, nuclear and acid-soluble fractions analogous to those now prepared.

Microsomal and supernatant fractions. In control rats (Fig. 1), maximal labelling occurred at about 30 hr. with the supernatant fraction, and at about 40 hr. with the microsomal fraction, the labelling of the latter being low in the first few hours after the injection. The labelling of both fractions remained high for at least the next 3 days.

If the RNA of the supernatant fraction were the precursor of microsomal RNA, the descending portion of the specific-activity curve for the former should intersect that for the latter at its maximum (Zilversmit, Entenman & Fishler, 1943). The data for control rats in Fig. 1 are compatible with this possibility, the curves for percentage recovery being also a rough measure of specific activity since microsomal and supernatant fractions as now prepared contain similar amounts of RNA (about 8 mg./100 g. body wt.; cf. Reid, 1956; Reid *et al.* 1956).* Consideration is given below to the apparent plateau in the ascending portion of the curve for the supernatant fraction.

* In Table 3 of Part 3 (Stevens & Reid, 1956), the values refer, not to RNA, as incorrectly stated, but to RNAP.

Table 2. *Rechromatography of heterogeneous ribonucleotide fractions*

See Table 1 for abbreviations and for method of expressing results. Fraction nos. are those indicated in Table 1.

Fraction no.	Expt. no.	UMP	IMP	UDP	UDP-glucuronic acid, *CTP*	UTP	GDP	GTP
				Control rats				
	3*c*	9·0	5·4	—	—	—	—	—
	4*c*	12·9	8·5	—	—	—	—	—
	5*c*	9·5	4·5	—	—	—	—	—
				Adrenalectomized rats (2–3 weeks after operation)				
(5)	3*a*	5·2	4·6	—	—	—	—	—
	4*a*	4·6	6·5	—	—	—	—	—
	4*a*′	4·7	4·7	—	—	—	—	—
	5*a*	4·2	8·2	—	—	—	—	—
				Adrenalectomized rats (3 days after operation)				
	5*a*′	12·2	—	—	—	—	—	—
				Control rats				
	3*c**	1·3	—	0·2	6·0	—	—	—
	4*c*	0·9	—	2·0	11·7	—	—	—
	5*c*	0·4	—	2·5	17·5	—	—	—
(11)				Adrenalectomized rats (2–3 weeks after operation)				
	4*a*′	0	—	0·9	3·8	—	—	—
	5*a*″	1·8	—	0·6	2·0	—	—	—
				Control rats				
	5*c*	0	—	0·3	—	3·5	1·5	0·9
(13)				Adrenalectomized rats (2–3 weeks after operation)				
	5*a*	0	—	0·3	—	0·9	0·8	—
	5*a*″	0	—	0·4	—	0·9	<0·2	0·5

* This experiment, in which the conditions were not optimum, has been disregarded in assessing the effect of adrenalectomy on UDP and UDPglucuronic acid.

The labelling of the supernatant fraction was higher with adrenalectomized rats than with control rats at all times up to 13 hr., and lower at all subsequent times. However, the labelling of the microsomal fraction was lower in adrenalectomized rats at all times, and especially after 13 hr. or longer. Even if allowance is made for the previous finding (Reid, 1956; Stevens & Reid, 1956) that supernatant fractions from adrenalectomized rats contain somewhat more RNA than those from controls, the curves for adrenalectomized rats are also compatible with the hypothesis that the RNA of the supernatant fraction is the precursor of microsomal RNA.

One assumption underlying this postulate is that the microsomal and supernatant fractions are each homogeneous with respect to RNA. It appears from the following experiments that the supernatant fraction is not entirely homogeneous in this respect.

Subfractions obtained from supernatant fraction. The results for subfractions obtained by ultracentrifuging are shown in Fig. 2. It is evident that there is no striking difference in the time course of labelling between the sedimented ('pellet') RNA (this being about one-half of the original RNA) and the non-sedimented ('supernatant') RNA, and that the subfractions are equally affected by adrenalectomy except, perhaps, with respect to the labelling at 20 hr.

In the next experiment (Fig. 3), $(NH_4)_2SO_4$ was added to 35 % of saturation, and then to 55 % of saturation. The results were somwhat variable, but it does appear that at short time intervals, in contrast with long time intervals, the precipitate obtained at 35 % of saturation becomes labelled as rapidly as the other subfractions and has a higher specific activity (cf. RNA values in Table 3). There is no indication that adrenalectomy affects one of the fractions selectively with respect to labelling. However, the precipitate obtained between 35 and 55 % of saturation may be particularly affected with

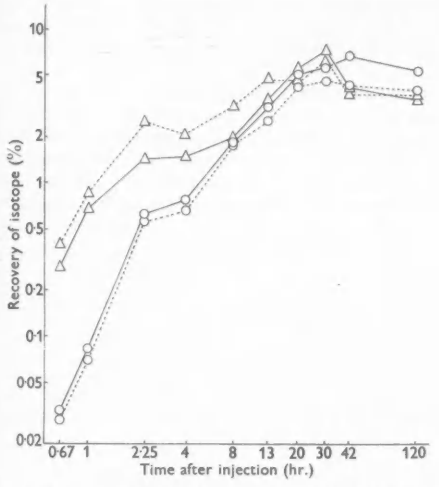


Fig. 1. Incorporation of injected orotic acid into the RNA of the microsomal fraction (○) and supernatant fraction (△) in control rats (——) and adrenalectomized rats (- - - -). Total no. of rats, 59.

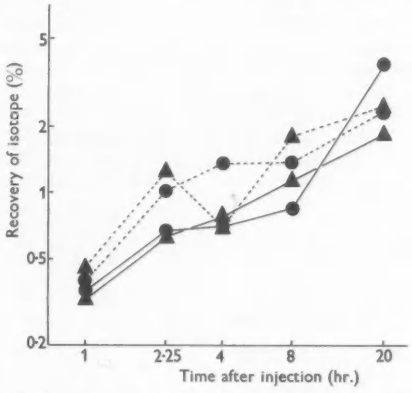


Fig. 2. Incorporation of injected orotic acid into the RNA of subfractions separated from supernatant fraction, namely ultracentrifugal pellet (●) and ultracentrifugal supernatant (▲), in control rats (——) and adrenalectomized rats (- - - -). Total no. of rats, 22.

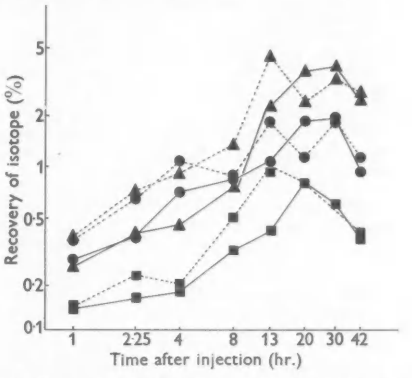


Fig. 3. Incorporation of injected orotic acid into the RNA of subfractions separated from supernatant fraction, namely precipitate obtained by ammonium sulphate at 35 % of saturation (●), precipitate obtained from 35 to 55 % (▲), and supernatant (■), in control rats (——) and adrenalectomized rats (- - - -). Each point represents one experiment; total no. of rats used in preparation of supernatant fraction 'pools', 28.

respect to the amount of RNA (Table 3), this being increased by 16%, whereas the average increases for the first precipitate and for the final supernatant were 10 and 2·5% respectively.

Other experiments (not illustrated) were performed with three concentrations of $(NH_4)_2SO_4$, namely 33, 38 and 55% saturation. The activity values showed the same trends as in the above experiments, the second precipitate being intermediate in behaviour between the first and third precipitates.

Barnum & Huseby (1950) mention preliminary experiments in which the RNA of the supernatant fraction, from mice given ^{32}P, was fractionated by $CaCl_2$ (0·4%) or by isoelectric precipitation: 'It was invariably possible to obtain a small non-precipitable fraction with a comparatively high relative specific activity.... The reproducibility of these fractions was poor, but it is possible to conclude that the supernatant PNA [i.e. RNA] contains a small fraction whose relative specific activity initially increases much more rapidly than does that of the microsomal PNA.' Accordingly, $CaCl_2$ has now been employed, with the results shown in Fig. 4. Since little more than one-half of the original RNA was precipitated (Table 3), presumably less than in the experiments mentioned by Barnum & Huseby, the curves representing percentage recovery are also a rough measure of specific activity. The data for both adrenalectomized and control rats do in fact show a difference between the precipitable RNA and the non-precipitable RNA, the specific activity of the latter being about double that of the former at the shortest time interval (40 min.) and half that of the former at long time intervals (20 and 42 hr.). Since, however, the peak of labelling occurred at the same time for both subfractions, as for the precipitates obtained with $(NH_4)_2SO_4$, it cannot be concluded that one is the direct precursor of the other.

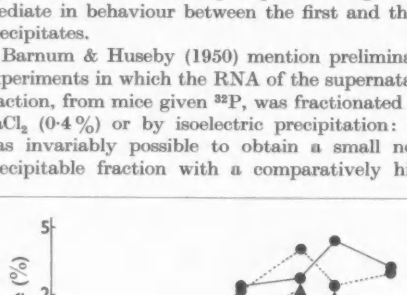


Fig. 4. Incorporation of injected orotic acid into the RNA of subfractions separated from supernatant fraction, namely precipitate (●) and supernatant (▲) obtained by addition of $CaCl_2$ to 0·1% (w/v), in control rats (——) and adrenalectomized rats (- - - -). Each point at 13 hr. represents two experiments, and the other points each represent one experiment; total no. of rats used in preparation of supernatant fraction 'pools', 25.

The data of Table 3 show that an increase in the amount of RNA after adrenalectomy occurs more consistently with the precipitable subfraction than with the non-precipitable subfraction.

The specific activity of the non-precipitable RNA at 40 min. after the injection was little altered by the use of lower final concentrations of $CaCl_2$ (0·06 or 0·075% in place of 0·1%). Concentrations higher than 0·1% did not increase the amount of RNA precipitated.

Isoelectric precipitation was performed in one experiment, with a short interval after isotope injection, but the specific activity of the non-precipitable RNA was no higher than that of the precipitable RNA, in contrast with the results mentioned by Barnum & Huseby (1950). The trial of alumina gel (C_γ) as an alternative means of fractionation was abandoned when similar values were found, at long or short intervals after isotope in-

Table 3. *Ribonucleic acid in subfractions separated from supernatant fraction of rat liver*

Values are expressed as mg. of RNA/100 g. body wt. The last column gives the mean differences (adrenalectomized rats minus controls) ± S.E. of differences (with no. of degrees of freedom in parentheses).

	Control rats: mean value	Adrenalectomized rats: mean difference from controls
$(NH_4)_2SO_4$ fractionation		
1st precipitate (35% of saturation)	2·7	+0·27±0·135 (6)
2nd precipitate (35% to 55% of saturation)	4·9	+0·79±0·291 (7)*
Supernatant	1·9	+0·05±0·123 (6)
$CaCl_2$ fractionation		
Precipitate (0·1% $CaCl_2$)	6·0	+0·82±0·339 (8)†
Supernatant	4·0	+0·53±0·459 (8)

* $P<5\%$. † $P<10\%$.

jection, for the ratio of the specific activity of the non-adsorbed RNA to that of the RNA eluted from the gel by 0·15 M-Na_2HPO_4 solution.

Nuclear fraction. Data for nuclear fractions, containing any cells left unbroken after the two treatments in the homogenizer, are given in Fig. 5. In view of the heterogeneity of the RNA in this crude fraction, no conclusions can be drawn from the recovery values, or even from specific activity values, concerning the possible role of nuclear RNA as the precursor of cytoplasmic RNA. It can, however, be concluded that the increase in the labelling of supernatant-fraction RNA after adrenalectomy is not attributable to more rapid entry of label into nuclear RNA with consequent faster transfer to the supernatant fraction.

In a few experiments, crude nuclear fractions were treated repeatedly with citric acid solution as in the experiments of McIndoe & Davidson (1952). With these partially purified nuclei, as with the crude fractions, there was no indication of an increase in activity after adrenalectomy at the times studied (1, 4 and 20 hr. after injection).

Acid-soluble fraction. The recovery of activity in this fraction, as shown in Fig. 5, was not consistently higher after adrenalectomy. Evidently adrenalectomy does not increase the proportion of injected orotic acid which enters the liver. It was of interest to know whether the pattern of labelling of the various constituents of the acid-soluble fraction is altered by adrenalectomy. Anion-exchange chromatography was therefore carried out with tissue excised 40 min. after injection of orotic acid in high dosage (Table 4). At this time the labelling of cytidine derivatives, which are present in relatively small amounts (Hurlbert & Potter, 1954), is low in comparison with that of uridine derivatives (Hecht & Potter, 1956). From the few experiments performed, it appears that the rate of conversion of orotic acid into uridine derivatives is unchanged or possibly somewhat decreased after adrenalectomy. Orotic acid given in low dosage is not detectable in the liver even 30 min. after injection, its conversion into uridine compounds being very rapid (Hurlbert & Potter, 1952, 1954). Its identification in the liver extracts now studied was based on its position (judged by the radioactivity and compared with that of authentic material) on the anion-exchange chromatogram and on a subsequent paper chromatogram with the propan-2-ol–water–HCl solvent of Wyatt (1951). In the former system orotic acid was not separable from ADP.

Labelling of liver deoxyribonucleic acid and of kidney ribonucleic acid. With the short time interval (135 min.) employed in the previous study (Reid *et al.* 1956), the labelling of liver deoxyribonucleic acid (DNA) was very low, and that of kidney RNA was too variable for any effect of adrenalectomy to be manifest. These aspects have now been reinvestigated, with the same dose (8 μc) but with a longer time interval (20 hr.). It was again found that the labelling of DNA was barely measurable, as also found by Hurlbert & Potter (1954), and that the labelling of the RNA in the whole kidney or in the supernatant fraction was very variable.

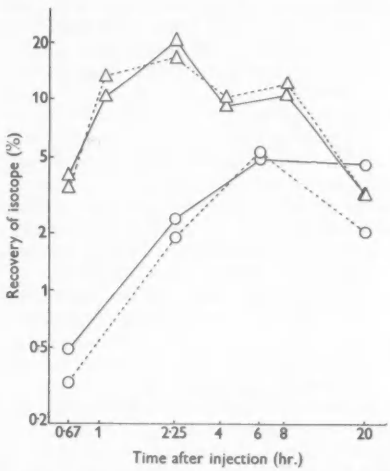


Fig. 5. Incorporation of injected orotic acid into the RNA of the nuclear fraction (○), and recovery of radioactivity in the acid-soluble fraction (△), in control rats (——) and adrenalectomized rats (- - - - -). Total no. of rats, 16 (nuclear fraction) or 30 (acid-soluble fraction).

Table 4. *Radioactivity of constituents of acid-soluble fraction of rat liver after large doses of orotic acid*

Rats were killed 40 min. after injection of [6-^{14}C]orotic acid. Techniques used for chromatography and for measuring radioactivity are described in the text; techniques in Expts. C and A differed in minor respects from those in C′ and A′. Values for the constituents (see Table 1 for abbreviations) represent percentage recovery of injected isotope.

	Expt.	UMP	Orotic acid	UDPacetyl-glucosamine, UDPglucose	UDP, UDPglucuronic acid
Control rats	C	2·4	0·4	2·6	—
	C′	1·45	1·5	1·6	0·8
Adrenalectomized rats	A	1·25	2·4	2·1	0·3
	A′	1·45	1·75	1·5	—

Incorporation of leucine in relation to time after injection of isotope

Whole cytoplasmic fraction. It was conceivable that a delayed absorption of injected DL-leucine after adrenalectomy was responsible for the decrease previously found (Reid *et al.* 1956) in the labelling of cytoplasmic protein. Since each of the cytoplasmic elements shared in this decrease, only the whole cytoplasmic fraction has now been reinvestigated. In the previous experiments, with an interval of 90 min. between injection and autopsy, the recovery of isotope (corrected for the presence of the 'inert' D isomer) in cytoplasmic protein was 9% in rats adrenalectomized 2 weeks previously, as compared with 10·2% in corresponding controls. With L-leucine, mean values of 7·1 and 7·25% respectively have now been found 40 min. after injection, and values of 9·2 and 10·7% have been found 15 min. after injection, the latter high values being perhaps a reflexion of day-to-day variation. It was anticipated that liver excised 15 min. after the injection would contain an appreciable amount of radioactivity in the form of free leucine; but the amount thus recovered was only about 0·1%, this amount being unaffected by adrenalectomy. It is, however, evident that the slight decrease in protein labelling after adrenalectomy is manifest even at relatively short time intervals.

Subfractions from microsomal fraction. Littlefield *et al.* (1955) have claimed that in the first few minutes after intravenous injection of a labelled amino acid, the activity of the microsomal protein which remains insoluble after deoxycholate treatment and which is rich in RNA is markedly higher than that of the solubilized protein. An attempt has now been made to ascertain whether adrenalectomy affects this initial labelling of the insoluble protein.

In preliminary experiments it was confirmed that a pellet consistently rich in RNA could be obtained by deoxycholate treatment of microsomal fractions, provided that the sedimented fractions were dispersed in a small volume of water before addition of the deoxycholate solution rather than dispersed directly in the latter. In the next experiment, liver was exised from control and adrenalectomized rats 3 min. after intravenous injection of labelled leucine, and microsomal fractions were rapidly isolated and treated with deoxycholate as in the experiments of Littlefield *et al.* (1955). In agreement with these authors, the specific activity of each pellet was higher than that of the corresponding supernatant (Table 5). However, the individual rats varied so widely that it was impossible to assess whether adrenalectomy had any effect on the labelling. There was no consistent effect of adrenalectomy on the yield or RNA content of the pellet or supernatant.

Incorporation of orotic acid and of leucine in relation to time after adrenalectomy

In the following experiments, adrenalectomized rats were studied at different times after operation; in isotopic experiments the time after injection of isotope was standardized as stated in the Experimental section.

Amount and labelling of ribonucleic acid. It is evident from Fig. 6 that the effect of adrenalectomy in increasing the amount of RNA in the supernatant fraction is not manifest 1 day after operation, but is fully manifest at 3 days. The increase in the incorporation of orotic acid into the RNA of the supernatant fraction is already manifest at 3 days, but continues to increase with time. On the other hand,

Table 5. *Labelling of microsomal subfractions 3 min. after intravenous injection of leucine*

Values represent counts/min./[100 − (% of RNA)] after injection of 12 μc of DL-[1-^{14}C]leucine/250 g. body wt., and are an arbitrary measure of the specific activity of the protein (the counting being at infinite thickness).

		Deoxycholate pellet	Deoxycholate supernatant
Control rats	Intact	2·4	2·2
	Intact	0·55	0·12
	Sham-operated	0·53	0·17
Adrenalectomized rats		2·4	0·34
		2·2	0·66
		0·57	0·13
		2·3	1·4

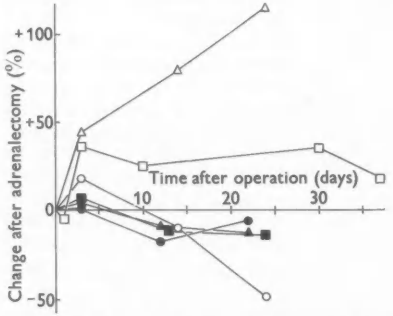


Fig. 6. Changes after adrenalectomy, related to time after operation: □, amount of RNA (/100 g. body wt.) in the supernatant fraction; △, incorporation of orotic acid into the RNA of the supernatant fraction; ○, incorporation of orotic acid into the RNA of the microsomal fraction; ■, amount of protein (/100 g. body wt.) in the microsomal fraction; ●, incorporation of leucine into protein of microsomal fraction; ▲, incorporation of leucine into protein of whole cytoplasmic fraction. The total nos. of rats were respectively 55, 22, 22, 30, 24 and 23.

the incorporation into the microsomal fraction is undiminished after 3 days, but subsequently decreases.

Amount and labelling of protein. Re-examination of the data of Reid *et al.* (1956) showed that adrenalectomy had consistently decreased the amount of microsomal protein as judged by the yield of the residue remaining after removal of acid-soluble material and lipids. As is now shown (Fig. 6), both this decrease and the decrease in the incorporation of leucine into the protein of the microsomes or the whole cytoplasm become manifest gradually, no decrease occurring only 3 days after operation.

Catabolism of ribonucleic acid

The possible effect of adrenalectomy on the catabolism of RNA cannot readily be assessed from the data of Fig. 1, the amount of RNA and its apparent rate of synthesis being themselves affected by adrenalectomy. The following approach was adopted. Adrenalectomy was performed 8 hr. after injection of isotope, presumably without effect on the time or extent of maximal labelling since, as was shown in Fig. 6, the amount of RNA in the supernatant fraction is not increased 1 day after operation. During the following 2 days the amount of this RNA rises to a maximum. If this rise were the consequence of a decrease in the catabolism of RNA, supernatant fractions prepared from the adrenalectomized rats after a few days' interval should have retained their labelling more fully than those from controls.

Table 6. *Catabolism of ribonucleic acid shortly after adrenalectomy*

Experimental rats were adrenalectomized, and the control rats sham-operated, 8 hr. after injection of 2·3 μc of orotic acid; the rats were killed after 5 days. Values represent percentage recovery of injected isotope.

	Microsomal fraction	Supernatant fraction
Control rats	6·9	3·75
	4·2	3·45
	5·0	3·2
Adrenalectomized rats	4·95	2·7
	4·1	2·35
	4·9	3·95

As is shown in Table 6, the labelling of the supernatant fraction after 5 days was no greater for the adrenalectomized rats than for the controls. The labelling of the microsomal fraction was also little affected, as would be expected in view of the observations that adrenalectomy does not affect either the amount of microsomal RNA (Reid, 1955), or its labelling shortly after operation (Fig. 6).

Effect of massive doses of orotic acid on liver ribonucleic acid levels

It is possible that orotic acid, or a uridine compound derived therefrom, is a limiting factor in RNA synthesis. To test this possibility, analyses for RNA were performed on livers from intact rats given massive doses of unlabelled orotic acid. Table 7 shows that this treatment appeared to increase markedly the amount of acid-soluble RNA precursors, as judged by the ultraviolet-light extinction at 260 mμ. The amount of RNA in the supernatant fraction was slightly increased, but that in the liver as a whole tended to decrease. Preliminary chromatographic studies with the acid-soluble fractions indicated that the increase in extinction was largely attributable to an increased amount of non-acidic material, possibly nucleosides.

A possible interpretation of these data is that the increase in supernatant-fraction RNA occurred only by virtue of enhanced breakdown or depressed formation of RNA elsewhere in the cell. Although the amount of RNA in the supernatant fraction did increase in response to administration of a uridine precursor, it is nevertheless possible that the incorporation of uridine nucleotides into RNA in normal rats is to some extent limited by the amount of the enzyme or enzymes concerned.

DISCUSSION

It is probable, although not proven for liver, that nucleic acids are formed at least in part from nucleoside 5′-diphosphates (pyrophosphates) or 5′-triphosphates (Grunberg-Manago, Oritz & Ochoa, 1955; Kornberg, Lehman, Bessman & Simms, 1956). Potter, Hecht & Herbert (1956) have achieved synthesis of liver RNA *in vitro* with ^{14}C-labelled orotic acid or UMP as precursor, having shown in

Table 7. *Effect of massive doses of orotic acid on liver-ribonucleic acid levels in intact rats*

The last column gives the mean differences (treated rats minus controls) ± S.E. of differences (with no. of degrees of freedom in parentheses).

	Control rats: mean value	Rats treated with orotic acid: mean difference from controls
Extinction at 260 mμ of acid-soluble fraction, diluted so that 1 l. is equivalent to 100 g. body wt.	0·264	+0·099 ± 0·0196 (18)*
RNA, mg./100 g. body wt. Whole liver	34·0	−3·1 ± 2·52 (17)
RNA, mg./100 g. body wt. Supernatant fraction	7·0	+1·0 ± 0·46 (18)†

* $P \ll 0{\cdot}1\%$.

† $P < 5\%$.

earlier experiments that liver contains a 'pool' of uridine nucleotides which rapidly become uniformly labelled after injection of orotic acid (Hurlbert & Potter, 1954; Schmitz, Hurlbert & Potter, 1954). The view that orotic acid, despite its apparent absence from normal liver (Hurlbert & Potter, 1952, 1954), is a physiological precursor of uridine nucleotides is supported by evidence from other laboratories (Lieberman, Kornberg & Simms, 1955; Hurlbert & Reichard, 1955; Wu & Wilson, 1956). Orotic acid is also a precursor of cytidine nucleotides, although the study of this conversion is handicapped by the difficulty of analysing the relatively small amounts of these nucleotides in liver (Hurlbert & Potter, 1954).

It has now been shown that at least some of the uridine nucleotides are markedly decreased in amount 2–3 weeks after adrenalectomy. That this observation does not hold for adenosine or guanosine nucleotides is suggested by the data for AMP and GMP, the amount of the former being actually greater after adrenalectomy. The changes in uridine nucleotide levels may have wide implications with respect to processes such as glucuronide synthesis. Here it need only be pointed out that if uridine nucleotides are in fact immediate precursors of supernatant-fraction RNA, as is discussed below, an increased incorporation of injected orotic acid into this RNA does not signify an increased rate of synthesis of RNA unless the increase in incorporation outweighs the decrease in the uridine nucleotide pool. Consideration of Table 2, together with Fig. 6, suggests that the rate of synthesis of supernatant-fraction RNA 2–3 weeks after adrenalectomy is increased not by about 100%, as Fig. 6 would suggest, but by less than 30%. That some increase does in fact occur is indicated by the data obtained 3 days after adrenalectomy, as is discussed below. (If supernatant-fraction RNA is formed, not from nucleotides but from nuclear RNA, the increased labelling of supernatant-fraction RNA may truly reflect increased synthesis, since adrenalectomy tends to increase, rather than diminish, the amount of RNA in the nuclear fraction [Reid, 1956].)

Obviously there may be other variables which could affect the interpretation of incorporation values in terms of rates of RNA synthesis. Adrenalectomy might conceivably have altered the rate or extent of the conversion of injected orotic acid into uridine nucleotides in liver. However, the possibility that adrenalectomy increases the amount of isotope entering the liver is ruled out by the results for the acid-soluble fraction (Fig. 5), the radioactivity of which is largely attributable to uridine nucleotides (Hurlbert & Potter, 1954). The results for radioactive uridine nucleotides in this fraction (Table 4), 40 min. after injection of orotic acid in a high dosage such that free orotic acid could be detected in the liver, suggest that the formation of uridine nucleotides is slowed rather than accelerated by adrenalectomy. Evidently, increased recovery of labelled material in supernatant-fraction RNA after adrenalectomy is not a consequence of a rise in the number of radioactive molecules in the acid-soluble fraction.

Since the amount of RNA in the supernatant fraction has already risen to a maximum 3 days after adrenalectomy (Fig. 6), an increased rate of synthesis of this RNA 2–3 weeks after adrenalectomy must be balanced by an increased rate of catabolism. This conclusion is compatible with the data of Fig. 1 and with data for ribonuclease levels (Reid & Stevens, 1956; Stevens & Reid, 1956) which will be discussed in a later paper.

It does appear that the increase in the amount of RNA occurring as an initial effect of adrenalectomy is attributable to increased synthesis rather than to decreased catabolism (Fig. 6 and Table 6), the uridine nucleotide pool being then elevated (with no increase in its labelling). This increased synthesis might be due to an increase in the amount of the enzyme(s) concerned in the formation of RNA from ribonucleotides. Daoust & Cantero (1955) suggest that 'the rate-limiting step in the synthesis of nucleic acids is not the formation of free nucleotides but rather the building up of nucleic acids from individual nucleotides'. The data of Table 7 are not incompatible with this view.

As Hurlbert & Potter (1954) have pointed out, the time course of labelling of the different fractions (cf. Figs. 1, 5) is compatible with the view that nuclear RNA is formed from acid-soluble ribonucleotides, but does not suggest a direct relationship between the latter and cytoplasmic RNA as has been assumed in the above discussion. The suggestion that nuclear RNA is the direct precursor of supernatant-fraction RNA (Jeener & Szafarz, 1950) is not supported by observations from other laboratories (Barnum, Huseby & Vermund, 1953; Smellie, McIndoe, Logan, Davidson & Dawson, 1953; Sacks & Samarth, 1956; Brachet, 1956), although Smellie *et al.* recognize the possibility that nuclear RNA contains several constituents, one of which may be such a precursor. It is, however, possible that the supernatant fraction contains at least two types of RNA, one of which is in fact formed directly from acid-soluble ribonucleotides.

It has now been found that subfractions differing in the time course of labelling can be separated from the supernatant fraction, in accordance with preliminary experiments mentioned by Barnum & Huseby (1950). Logan & Smellie (1956) have independently used ammonium sulphate to obtain subfractions which, in this instance, differed in their ability to donate ^{32}P-labelled RNA to the nucleus *in vitro*. Studies in which RNA was isolated at a fixed time after isotope injection and was deliber-

ately degraded, the products differing in specific activity values, have been reported by Sacks & Samarth (1956), whose interpretation of the results is, however, open to question, and by Moldave & Heidelberger (1954), who found 'intramolecular heterogeneity' with respect to the labelling of RNA phosphate (and guanine?) but not, in the supernatant fraction, with respect to that of adenine and pyrimidines after injection of labelled glycine and orotic acid.

The differences in labelling between the subfractions in the present experiments, together with the 'plateau' in the ascending portion of the curve for the whole supernatant fraction (Fig. 1), suggest the presence of two types of RNA, one of which may be the precursor of the other, the labelling of both types being enhanced by adrenalectomy. More rigorous fractionation will be required if the criteria of Zilversmit *et al.* (1943) for a precursor–product relationship are to be met. With orotic acid there is the inherent difficulty that RNA cytosine, as well as uracil, becomes labelled at long time intervals after isotope injection (Hurlbert & Potter, 1954); in the nuclear fraction from regenerating liver the RNA pyrimidines have become equally labelled at 12–24 hr. after isotope injection (Hecht & Potter, 1956).

Despite the latter complication and the probable heterogeneity of the RNA in each of the cellular elements, some evidence has been obtained in support of the postulate that microsomal RNA is formed from supernatant-fraction RNA (Jeener & Szafarz, 1950; Barnum *et al.* 1953). Whereas the specific activity values for microsomal and supernatant fractions after injection of ^{32}P showed no maxima in the experiments of Barnum *et al.* (1953) and similar maxima in the experiments of Smellie *et al.* (1953), the microsomal fractions now studied have shown a maximum, albeit broad and late, which is intersected by the descending portion of the curve for the supernatant fraction. (As has been pointed out above, the curves of Fig. 1 can be regarded as representing specific activity as well as percentage recovery.) Moreover, in the experiment of Table 7 the preferential increase in supernatant-fraction RNA suggests that the synthesis of this RNA precedes that of RNA elsewhere in the cytoplasm.

Closer consideration of the isotopic data from the kinetic aspect must await study of each fraction with respect to the amounts and specific activities of RNA cytosine and uracil. It may, however, be pointed out that at short time intervals after isotope injection the specific activity of the RNA in the supernatant fraction was somewhat increased after adrenalectomy, as in the previous experiments (Reid *et al.* 1956). The supposed translocation of RNA from the supernatant fraction to the microsomal fraction may therefore be retarded more than is suggested by the reduction in percentage of isotope recovered in the microsomal fraction.

The reduction in microsomal labelling, unlike the increase in the labelling of the supernatant fraction, is a late effect of adrenalectomy. From this observation and also from the data for short time intervals in Fig. 1, it can be concluded that the enhanced labelling of the supernatant-fraction RNA could not be due merely to a decrease in its supposed translocation to the microsomal fraction. Whatever the origin of microsomal RNA, it is evident that the lack of adrenal hormones affects the processes of cytoplasmic RNA synthesis at two separate points. That adrenalectomy also affects the synthesis of nuclear RNA is suggested by the results of Fig. 5, considered together with the fall in the amounts of uridine nucleotides (Tables 1 and 2); this fall in the pool of supposed precursors of nuclear RNA implies that the synthesis of this RNA is diminished more than is suggested by the slight diminution in the labelling of this RNA.

The decrease in microsomal labelling shows a striking parallelism, in its gradual onset after adrenalectomy, with the decreases in the incorporation of leucine into protein and in the amount of microsomal protein (Fig. 6). Since the rise in the labelling of supernatant-fraction RNA is much earlier, it is probable that at least some of this RNA, unlike the RNA in the microsomal fraction, is not directly concerned in protein synthesis. Admittedly it has not been proved in the present experiments that the small reduction in leucine incorporation truly signifies a fall in protein synthesis; but the absence of a significant effect of adrenalectomy on the pool of leucine in liver (Awapara, Skellenger & Manz, 1955) or on amino acid absorption by the liver (Awapara & Kit, 1954) argues in favour of this interpretation.

'Microsomal' material as prepared in some laboratories comprises both the microsomal fraction and the ultracentrifugal fraction as now prepared. The divergence between these fractions observed by Reid *et al.* (1956), with respect to the change in the incorporation of orotic acid after adrenalectomy, has now been confirmed (Figs. 1 and 2). A similar divergence has been observed by Jardetzky & Barnum (1956) in a study of RNA formation in regenerating liver.

SUMMARY

1. Adrenalectomy leads eventually to a marked decrease (exceeding 50 %) in the levels, in rat liver, of uridine 5′-phosphate, uridine 5′-diphosphate (-pyrophosphate) and its glucuronic acid derivative, and uridine triphosphate. There is also some decrease in uridine diphosphate acetylglucosamine

and possibly in uridine diphosphate glucose, but not in liver nucleotides in general.

2. Microsomal and supernatant fractions have been isolated from liver at different times after injection of labelled orotic acid. The results confirm that the labelling of the ribonucleic acid of the microsomal fraction is decreased after adrenalectomy and that of the supernatant fraction increased, and are compatible with the possibility that ribonucleic acid in the supernatant fraction is a precursor of microsomal ribonucleic acid.

3. Supernatant fractions containing labelled ribonucleic acid have been fractionated by several methods, two of which have indicated heterogeneity in this ribonucleic acid with respect to the time course of labelling. However, the labelling of the different subfractions was equally affected by adrenalectomy.

4. The labelling of nuclear and acid-soluble fractions, and of uridine nucleotides separated from the latter, shows no increase after adrenalectomy.

5. The labelling of microsomal ribonucleic acid, the labelling of cytoplasmic or microsomal protein with leucine as precursor, and the amount of microsomal protein are undiminished 3 days after adrenalectomy but decrease thereafter. The increase in the labelling of supernatant-fraction ribonucleic acid is already apparent 3 days after adrenalectomy, together with a rise in the amount of this ribonucleic acid which evidently occurs because of an increase in synthesis rather than a decrease in catabolism.

The experiments were commenced during the tenure of a Fellowship of the British Empire Cancer Campaign (E.R.); funds for the purchase of an ultraviolet-light spectrophotometer were also provided by the Campaign. The isotopic experiments were performed in the Radioisotope Laboratory of the Institute at Chalfont St Giles, Bucks, by courtesy of Professor J. A. V. Butler, F.R.S. Thanks are expressed to Mr R. T. Charles, Dr P. Cohn, Mr J. T. Nodes and Mrs J. Q. Tapley for assistance in various respects, and to Dr V. C. E. Burnop for preparing the labelled orotic acid. The study of the effect of large doses of unlabelled orotic acid was made at the suggestion of Dr A. L. Greenbaum of University College London. The investigation was supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Awapara, J. & Kit, S. (1954). *Endocrinology*, **55**, 186.
Awapara, J., Skellenger, W. & Manz, N. (1955). *Tex. Rep. Biol. Med.* **13**, 1.
Barnum, C. P. & Huseby, R. A. (1950). *Arch. Biochem.* **29**, 7.
Barnum, C. P., Huseby, R. A. & Vermund, H. (1953). *Cancer Res.* **13**, 880.
Brachet, J. (1956). In *Ionizing Radiations and Cell Metabolism, Ciba Foundation Symp.* p. 3. London: J. and A. Churchill.
Daoust, R. & Cantero, A. (1955). *Cancer Res.* **15**, 734.
Grunberg-Manago, M., Oritz, P. J. & Ochoa, S. (1955). *Science*, **122**, 907.
Hecht, L. I. & Potter, V. R. (1956). *Cancer Res.* **16**, 999.
Hurlbert, R. B. & Potter, V. R. (1952). *J. biol. Chem.* **195**, 257.
Hurlbert, R. B. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 1.
Hurlbert, R. B. & Reichard, P. (1955). *Acta chem. scand.* **9**, 251.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
Jardetzky, C. D. & Barnum, C. P. (1956). *Fed. Proc.* **15**, 282.
Jeener, R. & Szafarz, D. (1950). *Arch. Biochem.* **26**, 54.
Kornberg, A., Lehman, I. R., Bessman, M. J. & Simms, E. S. (1956). *Biochim. biophys. Acta*, **21**, 197.
Korner, A. & Debro, J. R. (1956). *Nature, Lond.*, **176**, 1067.
Lieberman, I., Kornberg, A. & Simms, E. S. (1955). *J. biol. Chem.* **215**, 429.
Littlefield, J. W., Keller, E. B., Gross, J. & Zamecnik, P. C. (1955). *J. biol. Chem.* **217**, 111.
Logan, R. & Smellie, R. M. S. (1956). *Biochim. biophys. Acta*, **21**, 92.
McIndoe, W. M. & Davidson, J. N. (1952). *Brit. J. Cancer*, **6**, 200.
Moldave, K. & Heidelberger, C. (1954). *J. Amer. chem. Soc.* **76**, 679.
Potter, V. R., Hecht, L. I. & Herbert, E. (1956). *Biochim. biophys. Acta*, **20**, 439.
Reid, E. (1955). *Nature, Lond.*, **175**, 461.
Reid, E. (1956). *J. Endocrin.* **13**, 319.
Reid, E., O'Neal, M. A., Stevens, B. M. & Burnop, V. C. E. (1956). *Biochem. J.* **64**, 33.
Reid, E. & Stevens, B. M. (1956). *J. Endocrin.* **14**, xli.
Sacks, J. & Samarth, K. D. (1956). *J. biol. Chem.* **223**, 423.
Schmitz, H., Hurlbert, R. B. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 41.
Schmitz, H., Potter, V. R., Hurlbert, R. B. & White, D. M. (1954). *Cancer Res.* **14**, 66.
Schneider, W. C. (1946). *J. biol. Chem.* **166**, 595.
Smellie, R. M. S., McIndoe, W. M., Logan, R., Davidson, J. N. & Dawson, I. M. (1953). *Biochem. J.* **54**, 280.
Spirtes, M. A. & Eichel, H. J. (1954). *Arch. Biochem. Biophys.* **53**, 308.
Stevens, B. M. & Reid, E. (1956). *Biochem. J.* **64**, 735.
Wu, R. & Wilson, D. W. (1956). *J. biol. Chem.* **223**, 195.
Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.
Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). *J. gen. Physiol.* **26**, 325.

The Biochemistry of Aromatic Amines

4. *O*-GLUCOSIDURONIC ACID DERIVATIVES OF 2-NAPHTHYLAMINE*

By E. BOYLAND and D. MANSON
Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, Fulham Road, London, S.W. 3

(*Received* 26 *February* 1957)

The metabolism of 2-naphthylamine is of particular interest because the amine is known to cause cancer of the bladder in man and dogs and the carcinogenic action is undoubtedly due to a metabolite excreted in urine. From the knowledge of the behaviour of the metabolites identified by earlier workers and by Boyland & Manson (1955) and Boyland, Manson & Orr (1957) it was argued (Boyland, 1956) that only one metabolite, 2-amino-1-naphthyl glucosiduronic acid, should be carcinogenic. Bladder cancer in mice has since been induced with this glucuronide (Allen, Boyland, Dukes, Horning & Watson, 1957). The preparation and properties of this and other 2-amino-naphthyl glucosiduronic acid derivatives are described in this paper.

MATERIALS

2-Naphthylamine, 2-acetamidonaphthalene, 2-acetamido-1-naphthol and 2-amino-6-naphthol hydrochloride were prepared as described by Booth, Boyland & Manson (1955). Calf-spleen β-glucuronidase (Viobin Laboratories, Monticello, Ill., U.S.A.) and bacterial β-glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) were employed for enzymic hydrolyses.

METHODS

Rats and rabbits were kept in metabolism cages and urine was collected daily. Rabbits were fed on cabbage, bran, rat cake and water, and rats on bread, rat cake and water. 2-Naphthylamine or 2-acetamidonaphthalene was injected intraperitoneally in daily doses of 0·5 g. in 10 ml. of arachis oil into rabbits and 0·05 g. in 1 ml. of oil into rats. Paper chromatography was carried out by upward development on Whatman no. 1 paper with butanol–propanol–aqueous 0·1 N-NH_3 (2:1:1, by vol.). Acetamido compounds were hydrolysed to amino compounds on the chromatograms by spraying with N-HCl and heating for 30 min. between glass plates at 70°. The colour reactions of acetamidonaphthyl glucosiduronic acids after hydrolysis under such conditions were characteristic of the aminonaphthyl glucosiduronic acids and not of the aminonaphthols. Reagents used in this work have been described by Booth *et al.* (1955). 2-Amino-1-naphthol was detected in solution by the green-coloured derivative which is formed on addition of NH_3. This derivative is extracted with benzene, in which solvent it is mauve (Liebermann & Jacobson, 1882). A Chromatolite lamp (Hanovia Ltd.) was used as a source of ultraviolet light. R_F values and colour reactions of the compounds isolated are given in Table 1. R_F values obtained by downward development in several solvent systems and colour reactions with nitrous acid followed by hexylresorcinol and with Ehrlich's reagent have been given by Booth *et al.* (1955).

RESULTS

Isolation of glucosiduronic acids

Both 2-amino-1-naphthol hydrochloride and the free base are toxic (LD_{50} in mice 25 mg./kg. body wt.) and so unsuitable as sources of 2-amino-1-naphthyl glucosiduronic acid. The administration of unsubstituted aromatic amines causes excretion of free glucuronic acid, which might interfere with the isolation of glucosiduronic acids. 2-Acetamido-1-naphthol was therefore used and the acetamido group of the glucosiduronic acid was hydrolysed by dilute sulphuric acid. Attempts to isolate 2-amino-1-naphthyl glucosiduronic acid from the urine of rats dosed with 2-naphthylamine were unsuccessful, although the acid could be detected in the urine by paper chromatography. Small amounts of 2-amino-6-naphthyl glucosiduronic acid were isolated from the urine of rabbits dosed with 2-amino-6-naphthol.

2-Acetamido-6-naphthyl glucosiduronic acid was isolated as its sodium salt from rabbit urine after administration of 2-acetamidonaphthalene. Rabbits deacetylate acetyl derivatives of arylamines relatively slowly, and when dosed with 2-acetamidonaphthalene rabbits excrete only small amounts of 2-amino-1-naphthylsulphuric acid and neither 2-naphthylsulphamic acid nor 2-amino-1-naphthyl glucosiduronic acid. 2-Acetamido-6-naphthyl glucosiduronic acid was, however, isolated from rabbit urine as the triacetyl methyl ester after injection of 2-naphthylamine. The glucosiduronic acid fraction was obtained by adsorption on charcoal and elution. If the charcoal was heated before use (cf. Corner, Billett & Young, 1954) the glucosiduronic acid was bound so that only small amounts were removed with cold or hot methanol, but it was eluted with aqueous phenol (cf. Dalgliesh, 1952). If the

* Part 3: Booth & Boyland (1957).

Table 1. *R_F values and colour reactions of 2-amino- and 2-acetamido-naphthyl glucosiduronic acids*

Conditions of hydrolysis are described in the text.

Compound	R_F in butanol–propanol–0·1N-NH_3 (2:1:1, by vol.)	Fluorescence	Colours with diazotized sulphanilic acid: Before hydrolysis	Colours with diazotized sulphanilic acid: After hydrolysis
2-Amino-1-naphthyl glucosiduronic acid	0·22	Blue–white	Pale yellow	Pale yellow
2-Acetamido-1-naphthyl glucosiduronic acid	0·25	None	None	Pale yellow
2-Amino-6-naphthyl glucosiduronic acid	0·15	Blue–white	Orange–red	Orange–red
2-Acetamido-6-naphthyl glucosiduronic acid	0·20	None	None	Orange–red

charcoal was not heated, cold methanol eluted only a little of the glucosiduronic acid; hot methanol removed more, but aqueous phenol was still necessary for complete elution. The glucosiduronic acid fractions contained 2-acetamido-6-naphthyl glucosiduronic acid, with small amounts of what is probably 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid. Evidence for the presence of the latter conjugate is discussed below.

2-*Acetamido*-1-*naphthyl glucosiduronic acid*

2-Acetamido-1-naphthol (0·3 g. in 10 ml. of arachis oil) was administered daily for 3 days to each of five rabbits (total dose 4·5 g.). The urine was adjusted to pH 4·0–5·0 with acetic acid and a saturated solution of lead acetate added. After removal of the precipitate by centrifuging, the supernatant was adjusted to pH 8·0 with aq. NH_3 (sp.gr. 0·88) and a saturated solution of basic lead acetate added until precipitation was complete. The precipitate was centrifuged off, washed with water, suspended in water and H_2S was passed in until precipitation of PbS was complete. The precipitate was filtered off and the filtrate concentrated *in vacuo*. On cooling, plates of 2-*acetamido*-1-*naphthyl glucosiduronic acid* were deposited, which after crystallization from water had m.p. 194–197°, $[\alpha]_D^{22}$ +2·4° in ethanol (*c*, 3·7) (Found: neutralization equivalent, 408; C, 52·4, 52·2; H, 5·8, 5·8; N, 3·4, 3·6%. $C_{18}H_{19}O_8N,2H_2O$ requires neutralization equivalent 413; C, 52·2; H, 5·6; N, 3·4%). On heating with 5N-HCl for 15 min. a compound was formed which after diazotization could be coupled with hexylresorcinol to give a red colour. It gave a positive reaction with naphthoresorcinol. After incubation with calf-spleen β-glucuronidase at pH 4·5 for 16 hr. at 37°, 2-acetamido-1-naphthol was identified by paper chromatography. A total of 1·5 g. of the compound was isolated, equivalent to 16% of the dose. The urine collected for a further 2 days yielded only a few milligrams of the glucosiduronic acid.

Examination of the untreated urine by paper chromatography showed that free 2-acetamido-1-naphthol and traces of 2-amino-1-naphthylsulphuric acid and 2-amino-1-naphthyl glucosiduronic acid were present, but no 2-acetamido-1-naphthylsulphuric acid could be detected.

2-*Amino*-1-*naphthyl glucosiduronic acid*

2-Acetamido-1-naphthyl glucosiduronic acid (1·0 g.) was heated on a water bath with 0·5N-H_2SO_4 (15 ml.) for 30 min., and the solution was treated with a small amount of charcoal, filtered and kept at room temperature overnight. Unchanged acetamido derivative (0·5 g.) separated and was filtered off; the filtrate was adjusted to pH 4·0 and evaporated in a vacuum desiccator until brown crystals began to appear. The solution was cooled to 5° overnight and the product filtered off. Recrystallization from water yielded small rosettes of prisms (100 mg., 11%) of 2-*amino*-1-*naphthyl glucosiduronic acid*, m.p. 178–180° (decomp.), $[\alpha]_D^{23}$ −155° in 0·1N-HCl (*c*, 0·175) (Found: C, 54·4; H, 5·8; N, 4·3, 4·2. $C_{16}H_{17}O_7N,H_2O$ requires C, 54·4; H, 5·4; N, 4·0%). The compound was soluble in water and readily soluble in ethanol. After hydrolysis by calf-spleen β-glucuronidase, 2-amino-1-naphthol was detected by the colour produced on shaking with aq. NH_3.

The acid hydrolysis gave some 2-acetamido-1-naphthol and for this reason the hydrolysis time was limited to 30 min. A guide to the optimum time of hydrolysis was obtained by examining samples by paper chromatography at intervals. Hydrolysis of the glucosiduronic acid linkage was slower than that of the acetamido group. Attempted hydrolysis with 2N-H_2SO_4 or 0·2N-HCl caused blackening and 2N-NaOH gave a red gum from which no 2-amino-1-naphthyl glucosiduronic acid could be isolated.

2-*Amino*-6-*naphthyl glucosiduronic acid*

Each of five rabbits received 2-amino-6-naphthol hydrochloride (0·45 g.), neutralized just before injection, for 4 days (total dose 7·2 g. of 2-amino-6-naphthol). The glucosiduronic acid fraction was separated by the lead acetate method and dissolved in the minimum of water, and the solution adjusted to pH 5·0. On cooling to 5° crystals of 2-*amino*-6-*naphthyl glucosiduronic acid* separated after 2 days. Recrystallization by solution in N-HCl and adjustment of the pH to 5·0 yielded 0·06 g. (0·4% of theory), $[\alpha]_D^{20}$ −76·8° in 0·1N-HCl (*c*, 0·625). The compound blackened and sintered at 250° (Found: C, 54·2; H, 5·55; N, 4·0. $C_{16}H_{17}O_7N,H_2O$ requires C, 54·4; H, 5·4; N, 4·0%). Paper chromatography showed that the mother liquors contained the acetamido derivative in addition to the amino compound.

2-*Acetamido*-6-*naphthyl glucosiduronic acid*

(*a*) *As the sodium salt.* 2-Acetamidonaphthalene was given daily to each of six rabbits for 7 days (total dose 16·8 g.). The collected urine was filtered (Ford's clarifying pad, grade F.C.B.) and adjusted to pH 7·0. Each day's urine was stirred for 0·5 hr. with about 50 g. of charcoal (British Drug Houses Ltd. activated charcoal, heated at 150° for about 0·5 hr. and cooled) and filtered. The almost colourless filtrate contained no detectable metabolites of 2-acetamidonaphthalene, but still gave a strong naphthoresorcinol reaction.

Preliminary experiments showed that elution of the charcoal with water or aq. 0·1N-NH_3 did not remove metabolites but that elution with hot methanol removed 2-acetamido-6-naphthol, 2-acetamido-6-naphthylsulphuric acid, a compound considered to be 2-acetamido-5:6-dihydro-

5:6-dihydroxynaphthalene and a trace of 2-acetamido-6-naphthyl glucosiduronic acid. Elution with 5% aqueous phenol removed the 2-acetamido-6-naphthyl glucosiduronic acid.

The charcoal was stirred with hot methanol (total vol. 3 l.) until all the first series of metabolites were extracted. The charcoal was then packed in a column above a layer of sand and treated with 5% aqueous phenol until the glucosiduronic acids were eluted, as indicated by paper chromatography. The eluate was evaporated to dryness and then kept in an evacuated desiccator over sodium hydroxide pellets for 24 hr. to remove the residual phenol. Solution of the residue in the minimum of water and the addition of several volumes of ethanol gave a hygroscopic precipitate (4 g.). (A sample of this preparation was incubated with bacterial β-glucuronidase in acetate buffer at pH 6·0 for 24 hr. at 37°; paper chromatography showed that 2-acetamido-6-naphthol and a little of the presumed 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene had been liberated.) After three crystallizations by solution in the minimum of hot water and addition of hot ethanol *sodium 2-acetamido-6-naphthyl glucosiduronate* (1·0 g.) was obtained as needles, m.p. 252–254° (decomp.), $[\alpha]_D^{25}$ −78·4° in water (*c*, 1·02) (Found: C, 49·5; H, 5·05; N, 3·2; Na, 5·8. $C_{18}H_{18}O_8NNa,2H_2O$ requires C, 49·65; H, 5·1; N, 3·2; Na, 5·3%). The yield was equivalent to 2·5% of the dose. The compound dissolved readily in water, giving a neutral solution, and was soluble in methanol and insoluble in ethanol. It gave a positive reaction with naphthoresorcinol and diazotized and coupled only after heating with 2N-HCl. After incubation with bacterial β-glucuronidase at pH 6·0 (acetate buffer) or pH 7·0 (phosphate buffer) 2-acetamido-6-naphthol was detected by paper chromatography but 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene was not present. The hydrolysis product was isolated, m.p. alone and mixed with an authentic specimen of 2-acetamido-6-naphthol, 221–223°.

Sodium 2-acetamido-6-naphthyl glucosiduronate (0·4 g.) on heating for 3 hr. at 100° in 5N-H_2SO_4 (10 ml.) gave 2-amino-6-naphthol, confirmed by the red colour given on diazotization and coupling with hexylresorcinol. The hydrolysis product was acetylated with acetic anhydride in pyridine and the diacetyl derivative, after recrystallization from ethanol, melted at 217–220°, alone and mixed with an authentic specimen of 2-acetamido-6-acetoxynaphthalene. Acidification of an aqueous solution of sodium 2-acetamido-6-naphthyl glucosiduronate gave no crystalline material and evaporation of the solution gave a yellow gum, soluble in water and ethanol, which could not be crystallized.

p-Toluidine 2-acetamido-6-naphthyl glucosiduronate was prepared by the addition of *p*-toluidine hydrochloride (0·1 g.) to a solution of the sodium salt (0·1 g.) in hot water (5 ml.). On cooling, needles separated which after recrystallization from water melted at 181–182° (decomp.), $[\alpha]_D^{24}$ −72·5° in water (*c*, 1·02) (Found: C, 62·0; H, 5·7; N, 5·8. $C_{25}H_{28}O_8N_2$ requires C, 62·0; H, 5·8; N, 5·8%).

In another experiment charcoal which was not preheated was used for adsorption and exhaustively extracted with hot methanol. The urine from rabbits dosed with 2-acetamidonaphthalene (28 g.) was divided into five portions, each approximately 2 l., which were each passed through columns of charcoal (250 g.). The columns were eluted with water (2 l.) and cold methanol (3 × 500 ml.). Indoxylsulphuric acid was identified in the methanol fractions. The columns were lagged and eluted with hot methanol (about 60°) in 500 ml. portions. Urinary phenols were eluted in the first three fractions. Twelve hot methanol washings were collected; the later washings contained very little 2-acetamido-6-naphthyl glucosiduronic acid. The hot methanol washings were combined, evaporated almost to dryness and the residue was dissolved in water and extracted several times with ether, followed by ethyl acetate. Ethyl acetate removes the compound which is thought to be 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene. The aqueous fraction was again evaporated to dryness and the residue dissolved in the minimum of butanol–propanol–water (2:1:1, by vol.) and sufficient Whatman cellulose powder added to form a paste. The paste was added to the top of a cellulose column (30 cm. × 6 cm.) prepared with the same solvent mixture and was slowly eluted over 5 days. Collection of fractions was guided by paper chromatography. 2-Acetamido-6-naphthol, 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene, 2-acetamido-6-naphthylsulphuric acid, an unidentified sulphuric ester and the glucosiduronic acid fraction were successively eluted from the column. Enzymic hydrolyses showed that this last fraction contained the glucosiduronic acids of 2-acetamido-6-naphthol and 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene, but mainly the former compound. The glucosiduronic acid fractions were combined and evaporated to dryness, the residue dissolved in the minimum of hot water and hot ethanol added until the solution was just cloudy. On cooling, rosettes of needles (3·5 g.) were formed, which after recrystallization gave sodium 2-acetamido-6-naphthyl glucosiduronate (1·5 g.) m.p. 252–254°. A further 1·0 g. of less pure material was obtained from the mother liquors and another 1·0 g. by elution of the charcoal with aqueous phenol. Attempts to isolate 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid by chromatography of the mother liquors or by fractional crystallization of the triacetyl methyl esters were not successful.

(*b*) *As the p-toluidine salt.* Urine from rabbits dosed with 2-acetamidonaphthalene (14 g.) was passed through a column of charcoal. The column was washed with water, with cold methanol which eluted relatively little of the glucuronide, and finally with aqueous phenol. The phenolic eluate was evaporated down, the residue dissolved in the minimum of water and 15 vol. of ethanol added. The precipitate (8·0 g.) was dissolved in water (70 ml.) and *p*-toluidine hydrochloride (12 g.) was added. After cooling overnight the *p*-toluidine salt of 2-acetamido-6-naphthyl glucosiduronic acid was collected and recrystallized twice from water to yield needles (2·3 g.), m.p. 181–182°, which did not depress the m.p. of the derivative prepared in (*a*).

(*c*) *As the triacetyl methyl ester.* Urine of rabbits dosed with 2-naphthylamine (total 10 g.) was passed through a charcoal column (150 g., not pretreated by heat). The column was washed first with water, then with hot methanol until the glucosiduronic acid fraction began to be eluted and then with aqueous phenol. The ethanol-insoluble material from the phenol eluate was dissolved in water, the pH adjusted to 5·0 and the solution evaporated to dryness in a desiccator. The residue was dissolved in methanol (100 ml.), treated with an ethereal solution of diazomethane (from 20 g. of nitrosomethylurea) and kept overnight at 5°. After filtration and concentration *in vacuo* the residue was dissolved in pyridine (10 ml.) and acetic anhydride (10 ml.). The solution was kept overnight and poured into water, and the precipitate

collected. After two crystallizations from aqueous ethanol *methyl (2-acetamido-6-naphthyl tri-O-acetylglucosid)uronate* was obtained as needles (0·7 g.), m.p. 205–206°, $[\alpha]_D^{24}$ – 28·6° in ethanol (*c*, 0·28) (Found: C, 58·1; H, 5·0; N, 2·7. $C_{25}H_{27}O_{11}N$ requires C, 58·0; H, 5·3; N, 2·7%). Although the original crude solid appeared to contain 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid no crystalline triacetyl methyl ester of this could be isolated.

Isolation of allantoin

The first and second cold methanol eluates from charcoal columns (see p. 277) were combined and the solution was concentrated to 30 ml. On cooling, prisms (1·5 g. from 10 l. of urine) separated, m.p. after three crystallizations from water 234–236° (decomp.), alone and mixed with an authentic specimen of allantoin (Found: N, 35·6. Calc. for $C_4H_6O_3N_4$: N, 35·4%). The compound had an R_F of 0·1 in butanol–propanol–0·1 N-NH_3, identical with that of allantoin. The spot gave a yellow colour with Ehrlich's reagent and a green colour with sodium hypochlorite (5% in water) after spraying with phenol (5% in 95% ethanol). On heating either the isolated substance or the authentic allantoin in 5 N-HCl with naphthoresorcinol it gave a cherry-red colour, extractable by ether. Asher (1910) described this colour reaction of allantoin and the adsorption of allantoin by charcoal. Corner & Young (1955) described the formation of a reddish purple colour from an unidentified constituent with naphthoresorcinol during the estimation of glucuronic acid in rat urine, but the colour did not interfere with the estimations. It is possible that allantoin was a contributory factor to the formation of this pigment. Under the conditions of the Dische (1947) method of estimating glucuronic acid, allantoin gave a blue–green colour.

The O-glucosiduronic acids formed in the metabolism of 2-naphthylamine

After rats and rabbits had been dosed with 2-naphthylamine the urine of each species was examined by paper chromatography for the presence of glucosiduronic acids. 2-Amino-1-naphthyl glucosiduronic acid was detected in the urine of both species, but was more abundant in that of the rat. Rat urine (2 ml.), diluted with an equal volume of 0·2 M-acetate buffer (pH 4·5), was incubated with calf-spleen β-glucuronidase at 37° for 2 days. 2-Amino-1-naphthol was liberated and identified by the ammonia–benzene test. A control urine sample gave no colour reaction to this test. A fraction containing the compound could be obtained by precipitation of the urine of rats or rabbits by basic lead acetate.

2-Amino-6-naphthyl glucosiduronic acid was detected in rat urine but not in rabbit urine. The compound was precipitated from rat urine by basic lead acetate. After decomposition of the lead salt by hydrogen sulphide a sample of the glucosiduronic acid fraction so obtained gave 2-amino-6-naphthol on incubation with calf-spleen β-glucuronidase. Rabbit urine, but not rat urine, contained a compound (R_F 0·2) which was non-fluorescent and which was not diazotizable until after treatment with acid at 70°. It corresponded in its reactions to 2-acetamido-6-naphthyl glucosiduronic acid. Paper chromatography of the urine after it had been heated for 15 min. with an equal volume of 5 N-hydrochloric acid gave a new spot which corresponded in R_F and colour reactions to 2-amino-6-naphthyl glucosiduronic acid. Although only one glucosiduronic acid appeared on hydrolysis there are two possibilities for the structure of its precursor. Both 2-acetamido-6-naphthyl glucosiduronic acid and 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid would give the amino compound on acid treatment. The glucosiduronic acid fraction of rabbit urine (50 ml.) was prepared by precipitation with basic lead acetate. After decomposition of the lead salt by hydrogen sulphide excess of the latter was removed by a current of air. The solution was adjusted to pH 6·5 and continuously extracted with ether for 18 hr. and then evaporated to 5 ml. in a desiccator at room temperature. After dilution with an equal volume of acetate buffer (pH 6·0) a sample of the solution was incubated with bacterial β-glucuronidase. Paper chromatography showed that two new spots were present. One corresponded to 2-acetamido-6-naphthol in R_F (0·92) and colour reactions. The other spot (R_F 0·81) was not fluorescent, did not react with diazotized sulphanilic acid, could not be diazotized and coupled with hexylresorcinol, but after treatment with acid at 70° the material in the spot could be diazotized (green colour with nitrous acid) and coupled with hexylresorcinol (red colour), and coupled with diazotized sulphanilic acid (mauve colour)—reactions identical with those of 2-amino-6-naphthol.

The material obtained by enzymic hydrolysis was also examined by two-dimensional chromatography, developing first with butanol–propanol–0·1 N-NH_3, then treating with acid at 70°, drying, and developing in the second direction with butanol–acetic acid–water (2:1:1, by vol.). Two spots corresponding in R_F and colour reactions to 2-amino-6-naphthol were present after the second development, one derived from the 2-acetamido-6-naphthol spot and the other from that of the unknown compound. The properties of this latter compound could be accounted for by the hydrolysis and dehydration of 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene. Acid-catalysed dehydration of cyclic dihydrodiols usually proceeds so that a hydroxyl group remains on the position which is normally attacked by electrophilic reagents (Badger, 1949). For example 3:4-dihydro-3:4-dihydroxychlorobenzene yields 4-hydroxychlorobenzene (Smith, Spencer & Williams, 1950) and 1:2-dihydro-1:2-dihydroxynaphthalene gives 1-naphthol (Young, 1947), although some 2-naphthol is also formed (Boyland & Sims, 1953). In the case

under consideration 2-acetamido-6-naphthol would probably be the major product (yielding the amino compound by simultaneous hydrolysis). The glucosiduronic acid spot with R_F 0·2 therefore represents a mixture of 2-acetamido-6-naphthyl glucosiduronic acid and 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene glucosiduronic acid. 2-Acetamido-1-naphthyl glucosiduronic acid was not detected in either rat or rabbit urines after injection of 2-naphthylamine or 2-acetamidonaphthalene. In support of this 2-acetamido-1-naphthol was not detected by paper chromatography after the enzymic hydrolysis of any of the glucuronide fractions.

DISCUSSION

Two *N*-glucosiduronic acid derivatives (2-naphthylamine *N*-glucosiduronic acid and 2-amino-1-naphthylsulphuric acid *N*-glucosiduronic acid) have been described as metabolites of 2-naphthylamine by Boyland *et al.* (1957). Four *O*-glucosiduronic acid derivatives are now described (three of which are metabolites of 2-naphthylamine) and evidence is given for the presence of a fourth *O*-glucosiduronic acid metabolite. It has been argued (Boyland, 1956) that only 2-amino-1-naphthyl glucosiduronic acid should be carcinogenic because the urinary β-glucuronidase would liberate 2-amino-1-naphthol from this. Bonser, Bradshaw, Clayson & Jull (1956) have shown that 2-amino-1-naphthol is carcinogenic when introduced into the bladders of mice. Using a similar technique Allen *et al.* (1957) have shown that 2-amino-1-naphthyl glucosiduronic acid induces bladder cancer in mice but that other metabolites, including 2-naphthylsulphamic acid, 2-naphthylamine *N*-glucosiduronic acid, 2-amino-6-naphthol and 2-acetamido-6-naphthol, are inactive. The other three of the glucosiduronic acid derivatives which have been isolated are now being tested for carcinogenic activity.

The occurrence of 2-amino-1-naphthyl glucosiduronic acid in urine of rats dosed with 2-naphthylamine is difficult to reconcile with the fact that rats do not develop bladder cancer when dosed with 2-naphthylamine, although rat urine contains much more β-glucuronidase than does human urine (D. C. Williams, personal communication). The β-glucuronidase should liberate the carcinogenic 2-amino-1-naphthol when the urine of rats dosed with 2-naphthylamine is allowed to stand. It is not liberated unless β-glucuronidase is added to this urine. This apparent anomaly is being investigated.

SUMMARY

1. 2-Acetamido-1-naphthyl glucosiduronic acid was isolated from the urine of rabbits dosed with 2-acetamido-1-naphthol.

2. The carcinogenic 2-amino-1-naphthyl glucosiduronic acid was prepared by hydrolysis of 2-acetamido-1-naphthyl glucosiduronic acid.

3. 2-Amino-6-naphthyl glucosiduronic acid was isolated from the urine of rabbits dosed with 2-amino-6-naphthol.

4. 2-Acetamido-6-naphthyl glucosiduronic acid was isolated as the sodium and *p*-toluidine salts from the urine of rabbits dosed with 2-acetamidonaphthalene and as methyl (2-acetamido-6-naphthyl tri-*O*-acetylglucosid)uronate from the urine of rabbits dosed with 2-naphthylamine.

5. Evidence is presented that a glucosiduronic acid of 2-acetamido-5:6-dihydro-5:6-dihydroxynaphthalene is excreted when rabbits are dosed with 2-naphthylamine or 2-acetamidonaphthalene.

6. Urine from rats and rabbits injected with 2-naphthylamine has been examined by paper chromatography. Rats and rabbits excrete 2-amino-1-naphthyl glucosiduronic acid. Rabbits excrete 2-acetamido-6-naphthyl glucosiduronic acid, but this compound was not detected in rat urine. Rats excrete 2-amino-6-naphthyl glucosiduronic acid, but rabbits do not. 2-Acetamido-1-naphthyl glucosiduronic acid was not detected in the urine of either species.

7. Allantoin (which gives a red colour with the naphthoresorcinol reagents) was isolated by adsorption on charcoal and elution with methanol.

The authors wish to thank Miss M. Biggs for technical assistance. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

REFERENCES

Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S. & Watson, G. (1957). *Brit. J. Cancer* (in the Press).
Asher, K. (1910). *Biochem. Z.* **26**, 370.
Badger, G. M. (1949). *J. chem. Soc.* p. 2497.
Bonser, G. M., Bradshaw, L., Clayson, D. B. & Jull, J. W. (1956). *Brit. J. Cancer*, **10**, 1956.
Booth, J. & Boyland, E. (1957). *Biochem. J.* **66**, 73.
Booth, J., Boyland, E. & Manson, D. (1955). *Biochem. J.* **60**, 62.
Boyland, E. (1956). *Bull. Soc. Chim. biol. Paris*, **38**, 827.
Boyland, E. & Manson, D. (1955). *Biochem. J.* **60**, ii.
Boyland, E., Manson, D. & Orr, S. F. D. (1957). *Biochem. J.* **65**, 417.
Boyland, E. & Sims, P. (1953). *J. chem. Soc.* p. 2966.
Corner, E. D. S., Billett, F. S. & Young, L. (1954). *Biochem. J.* **56**, 270.
Corner, E. D. S. & Young, L. (1955). *Biochem. J.* **61**, 132.
Dalgliesh, C. E. (1952). *Biochem. J.* **52**, 3.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Liebermann, C. & Jacobson, P. (1882). *Liebigs Ann.* **211**, 36.
Smith, J. N., Spencer, B. & Williams, R. T. (1950). *Biochem. J.* **47**, 284.
Young, L. (1947). *Biochem. J.* **41**, 417.

The Synthesis of Fluorocitric Acid and its Inhibition in Acetate

By R. A. PETERS and R. W. WAKELIN
Department of Biochemistry, Oxford, and A.R.C. Institute of Animal Physiology (Biochemistry Department), Babraham, Cambridgeshire

(*Received* 25 *February* 1957)

The theory that the citric acid formation induced by the poison fluoroacetate *in vitro* (Liébecq & Peters, 1949; Martius, 1949) and *in vivo* (Buffa & Peters, 1949) is due to the synthesis of a fluorotricarboxylic acid has been proved by the isolation of a crystalline fluorocitric acid (Peters, Wakelin, Buffa & Thomas, 1953) and by the observation that this substance inhibits the enzyme aconitase *in vitro* (Lotspeich, Peters & Wilson, 1952; Morrison & Peters, 1954). Two forms of aconitase are known, one present in mitochondria, the other obtainable in solution. Working with the soluble aconitase, Peters & Wilson (1952) and Morrison & Peters (1954) found that there is a competition between the inhibitor and the tricarboxylic acid substrates when the inhibition is induced by enzymically made fluorocitric acid. With synthetic fluorocitric acid (Rivett, 1953) the relations are more complex.

More information on the mechanism of synthesis of the fluorocitric acid from the fluorinated C_2 fragment was needed to place this upon a firm biochemical basis, partly for theoretical reasons and partly for the practical objective of understanding protective therapy. Bartlett & Barron (1947) advanced the hypothesis that fluoroacetate interfered with the metabolism of acetate, a hypothesis which was shown by Liébecq & Peters (1949) not to apply to the accumulation of citric acid, because they found conditions in which this accumulated in the presence of fluoroacetate without a change in acetic acid content. In connexion with the protective action of C_2 substances, it was early found (Hutchens, Wagner, Podolsky & McMahon, 1949) that acetate with or without ethanol protected against fluoroacetate poisoning in some animals. Farah, West & Angel (1950) also found that acetate prevented the pharmacological toxicity of fluoroacetate to preparations of rabbit small intestine, and there have been other instances since of such protective effects (see Peters, 1957). The failure of acetate in some animals might well be due to failure of the initial activation stage; it was later established (Chenoweth, Kandel, Johnson & Bennett, 1951) that monoacetin was highly protective, presumably owing to its liberation of active acetyl groups *in situ*. On similar lines Gitter, Blank & Bergman (1953) (see also Gitter, 1956) found that acetamide was protective; this might be expected to penetrate mitochondria.

None of these researches established whether acetate itself interfered at some active stage with the synthesis of fluorocitric acid. Peters (1952) reported the results of some preliminary experiments by R. W. Wakelin and himself on the effect of acetate on the synthesis of fluorocitric acid *in vitro*, and on the conditions leading to the best synthesis of fluorocitric acid using kidney preparations from the guinea pig. The experiments suggested that in fact it is acetate which interferes with the synthesis of fluorocitric acid from fluoroacetate. Subsequently these experiments were extended and more carefully controlled, partly because in this earlier work the apparent effects of acetate stopped short of the maximum. Difficulties were found on account of the small amounts of fluorocitric acid synthesized in experiments of this type; this makes it necessary to estimate the fluorocitric acid by the inhibition produced upon citrate metabolism. Estimations were done with kidney particles in the first instance, but later the results were confirmed with brain tissue.

METHODS

Reagents. Unless otherwise stated these were of A.R. quality. Sodium pyruvate was prepared by R. W. Wakelin in the laboratory. Synthetic fluorocitrate was a specimen prepared by Dr D. E. A. Rivett. Enzymic fluorocitrate was prepared by ourselves. Adenosine triphosphate (ATP) was from a commercial specimen of a barium salt, not more than 70% pure; tests, however, showed that it fulfilled the function required. Sodium fluoroacetate was from the Monsanto Chemical Co.; sodium malate was mostly from L-malic acid obtained commercially from U.S. sources, and not further purified. A test by Mr R. J. Hall showed that it was chromatographically homogeneous.

Estimations. Citric acid estimations were made by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1949).

Preparation and use of kidney particles. These were prepared from guinea-pig kidneys by our usual methods. To get full activity the details of these preparations are important. The tissue was first squashed thoroughly in a small ice-cold mortar with the pestle, and ice-cold KCl (1%) to about 10·0 ml. was added drop by drop at first, with constant stirring. If the initial additions were too large and not stirred in properly, the tissue appeared to coagulate and the

preparations were less active. The suspension was squeezed through muslin, and the homogenate centrifuged at 3500–4700 *g* for 30 min., after which the pellet of particles was separated and stirred into 1% KCl. Phosphate buffer (made from KH_2PO_4 neutralized with NaOH to pH 7·2) was added to 0·1 M immediately before pipetting into the flasks.

This particle suspension was reinforced with Mg^{2+} and ATP and exposed to various substrates with or without sodium fluoroacetate, being shaken in the water bath at 38° in 50 ml. Erlenmeyer flasks. At the completion of the experiments, the contents of the flasks were transferred quickly to thin test tubes and heated for 5 min. in a boiling-water bath, after which they were cooled and centrifuged. This procedure does not appear to alter the fluorocitric acid, and permits subsequent estimations of fluorocitrate. The supernatants from duplicate flasks were usually combined; this ensures sufficient solution for analysis where the citric acid content is low. In enzyme experiments of this nature it is preferable to shake each flask under exactly the same conditions rather than to make up a larger bulk. The effect of any possible changes in the enzymes during pipetting can be reduced by adding them to alternate flasks in the series. Suitable amounts of the respective supernatants from the first stage of the experiment were then taken for estimation of fluorocitric acid with a fresh set of kidney particles (second stage of the experiment), by using 10 μmoles of citric acid and 0·03 M-malonate to block re-entry of citrate breakdown products into the tricarboxylic acid cycle.

RESULTS

In our earlier series of experiments it was considered sufficient to set up controls with fluoroacetate but no substrate other than citrate; these showed no synthesis of fluorocitric acid when tested with the malonate-blocked particles as judged by interference with the metabolism of the citric acid in this second stage. Later experiments, however, demonstrated that even in the malonate-blocked kidney system of the second stage some synthesis of fluorocitrate from residual fluoroacetate could still take place from any residual amount of malate or similar substrate which might be still present in the supernatant from the first stage, and which escaped the malonate block. Table 1 illustrates this with the details of an experiment in which it is shown clearly that by itself fluoroacetate has no effect in the presence of malonate, i.e. the citric acid metabolism is blocked presumably at the succinate stage so that there is no synthesis of fluorocitric acid. However, the addition of small amounts of malate or fumarate are enough to give a marked synthesis of fluorocitrate. Hence there must be a control for traces of substrate as well as of fluoroacetate left from the first stage of the experiment. This control proved to be elaborate and difficult, especially where the amounts of fluorocitric acid formed were small. Experience proved that it was best to reduce the magnitude of the interference in the second stage by using not more than 1/15 of the 3·0 ml. from the first stage, and to make experiments with dummy mixtures of malate, fluoroacetate, acetate, ATP and Mg^{2+} ions so that the citrate formed in these could be also used in correction. With these corrections, it was no longer found that the apparent effect of acetate stopped short of the maximum; and this fact gives confidence that the controls were adequate. The two-stage experiment summarized in Table 2 is typical of several satisfactorily controlled experiments, which place the preliminary observations upon a solid basis.

In the first stage of the experiment, which is summarized in Table 2 (*a*), with kidney particles, L-malate and fluoroacetate, there was a 12–25% reduction of citrate accumulation in the presence of acetate. This effect is variable. In the second stage of the experiment with malonate [Table 2 (*c*)] it is seen that the acetate present in the first stage, even at 1·25 μmoles, has made a big difference in formation of fluorocitric acid as judged by the effects on citrate disappearance in the second stage, after correction as described in Table 2 (*b*). From Table 2 it is clear that the method is sensitive because even the small amounts of fluorocitrate formed in the presence of 2·5 μmoles of acetate (in

Table 1. *Synthesis from fluoroacetate of the inhibitor for citric acid metabolism in the presence and absence of fumarate and malate, in kidney particles blocked with malonate*

Twelve flasks were set up containing in each 1·9 ml. of enzyme particles from the kidneys of one guinea pig made up to contain 0·1 M-K_2HPO_4 neutralized to pH 7·2 with NaOH and malonate (0·0334 M), 0·1 ml. of $MgCl_2$ (4·0 μmoles), 0·1 ml. of ATP (1·2 μmoles), 0·4 ml. of sodium citrate (10 μmoles), additions of sodium fluoroacetate (0·2 ml., 8·0 μmoles), sodium fumarate or malate (0·1 or 0·2 ml.), with KCl (1%; 0·1–0·5 ml.) to make 3·0 ml. total volume. The flasks (in duplicate) were shaken for 30 min. at 38°, and the contents treated with trichloroacetic acid as usual.

Additions	Citrate estimated at end (μmoles/flask)	Diff. for substrate (μmoles)
None	0·94	—
Fluoroacetate	0·92	—
Fluoroacetate + fumarate (3·12 μmoles)	3·63	2·71
Fluoroacetate + fumarate (1·56 μmoles)	2·22	1·3
Fluoroacetate + malate (7·46 μmoles)	5·28	4·36
Fluoroacetate + malate (3·73 μmoles)	3·00	2·08

Table 2. *Summary of the two stages of an experiment with kidney particles (guinea pig)*

The first stage is designed for synthesis of fluorocitric acid, and the second for estimating the amounts synthesized. For further experimental details of second stage, see (*b*) and (*c*).

(*a*) *First stage*: kidney particles from one guinea pig were made up to 26 ml. in 1% KCl containing 0·1 M-phosphate buffer, pH 7·2. Portions were shaken in a volume of 3·0 ml. for 30 min. at 38°. 3·0 ml. contained 1·9 ml. of enzyme, 0·1 ml. of $MgCl_2$ (4·0 μmoles), 0·1 ml. of ATP (1·2 μmoles), 0·1 ml. of sodium L-malate (19 μmoles), 0·2 ml. of sodium fluoroacetate (20 μmoles), with additions of 0·1–0·2 ml. of sodium acetate, and KCl (1%; 0·4–0·6 ml.) to total volume. At the end of the experiment the flasks were quickly emptied into thin test tubes, which were kept cold on ice. Each test tube was then immersed in the boiling-water bath for 5 min. and centrifuged after cooling. The supernatant was available for the second stage, in which the fluorocitric acid formed was estimated. 1/15 of flask contents from the first stage was used for the second stage; for details see (*b*) and (*c*).

	First stage (malate as substrate)	Second stage (with citrate and malonate; no malate)		
		Citric acid* not metabolized (μmoles)		Estimated fluorocitrate per flask of first stage (μmole)
	Citrate formed (μmoles)	Per flask of 2nd stage	Per flask of 1st stage	
A. Fluoroacetate only†	8·12	2·20	33·0	0·171
B. Fluoroacetate + acetate (0·31 μmole)‡	7·14	1·95	29·3	0·152
C. Fluoroacetate + acetate (0·625 μmole)‡	6·95	1·62	24·3	0·126
D. Fluoroacetate + acetate (1·25 μmoles)†	6·57	0·67	10·0	0·052
E. Fluoroacetate + acetate (2·5 μmoles)†	6·16	0·015	0·2	0·001
No fluoroacetate or acetate	0·59	—	—	—
Fluorocitrate, 0·0143 μmole (3·0 μg.)	—	2·75	—	—
Fluorocitrate, 0·0286 μmole (6·0 μg.)	—	3·64	—	—

* As corrected. † Duplicate. ‡ Triplicate.

(*b*) *Second stage* (control).

Three mixtures were made up with the following composition:

	(i)	(ii)	(ii)
L-Malate (mg.)	2·5	2·5	2·5
Fluoroacetate (mg.)	2·0	2·0	2·0
Acetate	—	0·2 ml. (≡0·042 mg.)	0·2 ml. (≡0·34 mg.)
ATP (ml.)	0·1	0·1	0·1
Mg (ml.)	0·1	0·1	0·1
KCl–phosphate to a total volume (ml.) of	3·0	3·0	3·0

These were added to the control flasks of the same general composition as in the heading for the second stage below, and shaken under similar conditions:

Additions (ml.)	Citrate found after 30 min. (μmoles)	Diff. (μmoles)
Nil	0·938	—
0·2 (i)	1·213	+0·275
0·2 (ii)	1·344	+0·406
0·2 (iii)	1·025	+0·087
0·2 (i) + fluorocitrate (3 μg.)	3·96	+3·022 − (i) = 2·747
0·2 (i) + fluorocitrate (6 μg.)	4·84	+3·902 − (i) = 3·627

(*c*) *Second stage* (estimation of fluorocitrate formed in first stage with kidney particles blocked with malonate).

Kidney particles from two guinea pigs were made up in the usual KCl–phosphate mixture to which was added enough malonate to make this 0·0334 M. The flasks were shaken for 30 min. at 38°. Each flask contained, in a total volume of 3·0 ml., 1·9 ml. of enzyme solution, 0·1 ml. of $MgCl_2$ (4·0 μmoles), 0·1 ml. of ATP (1·2 μmoles), 0·4 ml. of sodium citrate (10 μmoles) and additions of 0·2 ml. with KCl (1%, 0·1–0·5 ml.) to bring to total volume.

Additions (0·2 ml.)*	Citrate found after 30 min. (μmoles)†	Diff. (μmoles)	After final correction‡
—	0·938	—	—
A	3·415	2·477	2·202
B	3·298	2·360	1·954
C	2·959	2·021	1·615
D	1·697	0·759	0·672
E	1·042	0·104	0·017

* 1/15 of contents of flasks from first stage. † Corrected for preformed citrate added in *A*–*E*.

‡ Corrected for the citrate formed from the small additions of malate and fluoroacetate as determined in the second stage (control).

3·0 ml. of the mixture) are enough to indicate a slight change of citrate catabolism in the mitochondrial preparation.

Errors. In this experiment, which is reported in full and which was the last to be done, the results show the inhibitory effect of acetate on the formation of fluorocitrate, the values for the latter giving a smooth curve when estimated by its effect on the metabolism of citric acid. With 2·5 μmoles of acetate there was almost complete suppression of fluorocitric acid formation, and even with a quarter of this amount the action was marked. These facts give confidence that the methods employed are sufficiently accurate, and this is reinforced by a large number of earlier experiments, all giving substantially the same answer. At the same time it is difficult to state the exact errors of such an experiment. The duplicate citric acid analyses are the average of figures differing by less than 0·02 μg. This will be magnified by multiplication and be a greater percentage error where less is estimated; but in a series of six duplicates the mean differed from the duplicate estimation by ± 5, 3, 5, 0·5, 0·25 and 3 %. From this it may be inferred that a single estimation may be 5 % out, and the mean ± 3·0 %. Hence a difference between two experiments will be of the order of ± 6·0 %. Therefore, when allowance is made for inhomogeneity of enzyme preparation, the final figures might not be more accurate than ± 10 %. Hence the drop in the citric acid not metabolized (Table 2) from 33 to 29·3 μmoles, taken by itself, is within experimental error. It becomes significant only when taken in conjunction with earlier experiments, all of which show effects at these relatively low concentrations of acetate.

It was interesting to know the amounts of fluorocitric acid corresponding to these inhibitions of citric acid metabolism. In Table 2 the inhibitory response was not linear with malonated kidney particles between 3 and 6 μg. of fluorocitric acid (enzymic) and that it was lower than the responses in kidney untreated with malonate, which might be expected to be 5–10 μmoles (Peters *et al.* 1953). This point was investigated in another experiment (Table 3) in which a direct comparison was made. The value with fluorocitric acid (0·0142 μmole) without malonate was 4·51, rather lower than the 5·0 μmoles which might be expected for the enzymic compound. With malonate the values were 2·75 and 4·27, 61 and 64 % of the values obtained in the absence of malonate. The value 2·75 μmoles for 0·0142 μmole was confirmed in another experiment, but the value found for 0·0285 μmole was lower. In another case, however, the relation was approximately linear up to 3·0 μg. of fluorocitric acid (0·0142 μmole). From the value 2·75, assuming a linear relation, provisional values of fluorocitric acid present were calculated which are shown in Table 2. It is clear that malonate reduces the accumulation of citrate in presence of fluorocitrate, perhaps due to inhibition of re-cycling of any metabolite of citrate which escapes the aconitase block. As the sodium malonate increases the tonicity of the media, in a control experiment the tonicity was increased by addition of NaCl, with little effect on citrate metabolism; hence an effect of change of tonicity can be excluded.

Brain 'particles'. In view of the complicated nature of the controls required for estimating fluorocitric acid with kidney particles, it appeared advisable to get some different proof of the same point, which was done as follows. In earlier papers (Liébecq & Peters, 1949; Peters & Wakelin, 1953) it had been proved that the pyruvate oxidase system present in brain particles (from the pigeon) under our conditions does not synthesize fluorocitric acid from fluoroacetate, and that fluorocitric acid blocks the metabolism of pyruvate with

Table 3. *Effect of fluorocitric acid (neutralized) on citric acid metabolism of kidney particles (from the guinea pig) in the presence and absence of malonate*

Kidney particles prepared from one guinea pig were suspended in 26 ml. of 1 % KCl containing ½ vol. of 0·5 M-phosphate buffer (KH_2PO_4 neutralized with NaOH to pH 7·2); to half sodium malonate was added so that each 3·0 ml. contained 15 mg. Twelve flasks were set up, each containing a total volume of 3·0 ml. made up of 1·9 ml. of kidney suspension, 0·1 ml. of $MgCl_2$ (4·0 μmoles), 0·1 ml. of ATP (1·2 μmoles), 0·4 ml. of citric acid neutralized (10 μmoles), KCl (1 %; 0·3–0·5 ml.), with additions 0–0·2 ml. of fluorocitrate (0·1 ml. ≡ 3 μg. of fluorocitric acid). Immediately after the final addition of enzyme the flasks were shaken at 38° for 30 min.; then 1 ml. of trichloroacetic acid (25 %) was added, and citrate estimated.

Additions	Citrate present (μmoles)	Diff. (μmoles)
Malonate present		
Citrate only	0·726	—
Citrate + fluorocitrate (0·0142 μmole)	3·48	2·75
Citrate + fluorocitrate (0·0285 μmole)	5·00	4·27
No malonate		
Citrate only	0·413	—
Citrate + fluorocitrate (0·0142 μmole)	4·92	4·51
Citrate + fluorocitrate (0·0285 μmole)	7·02	6·61

accumulation of citric acid and a diminution of oxygen uptake. Though less accurate this gives a good way to estimate fluorocitric acid in the presence of traces of fluoroacetate and malate or fumarate, because the latter must be present in any case in the pyruvate experiment where an addition of fumarate to the pyruvate is necessary for full activity. Accordingly, the first stage of the experiment with kidney particles, in which the system was arranged to synthesize fluorocitrate, was performed as before. The supernatant from this first stage was added in suitable quantities to a particle preparation from pigeon's brain, made by grinding according to our usual methods, in order to see how inhibitory it was to the citrate metabolism and oxygen uptake. The experiments in Table 4 demonstrate quite clearly that by this method there is no detectable synthesis of fluorocitric acid from fluoroacetate in the presence or absence of pyruvate; hence the method is valid in detecting fluorocitric acid in the presence of fluoroacetate. A point has been raised as to whether the addition of CoA would improve the synthesis. Reinforcement with CoA is essential in the choline acetylase system from pigeon brain (Balfour & Hebb, 1952; Gardiner & Whittaker, 1954). Such brains have been treated with acetone, which evidently alters permeability relations. Expt. 2 in Table 4 shows quite clearly that our particle preparations are not influenced by additions of CoA.

In the experiment given in Table 5, it is shown by the use of brain dispersions that the synthesis of fluorocitrate by kidney particles is inhibited by the presence of acetate.

DISCUSSION

Both types of experiment agree in demonstrating that 0·416 mM-acetate markedly inhibits the formation of fluorocitric acid from fluoroacetate (6·67 mM), and that even the effect of 0·2 mM-acetate is detectable. The advantage of the estimations with brain particles is that with these the formation of more fluorocitric acid during the estimation is

Table 4. *Failure of washed brain particles (pigeon) to synthesize fluorocitrate from fluoroacetate*

Experiment 1

In the first stage, pigeon-brain particles were exposed to fluoroacetate + pyruvate. In the second stage, extracts from this suspension were tested with fresh brain particles for the effect on citrate accumulation.

First stage. Brain-particle dispersions, from one pigeon, in KCl (1%) were centrifuged and made up in 14 ml. of KCl–phosphate mixture, pH 7·2, containing 0·05 M-phosphate. Six Erlenmeyer flasks were shaken in the bath at 38° for 40 min.; each contained 1·9 ml. of enzyme preparation, 0·1 ml. of $MgCl_2$ (4·0 μmoles), 0·1 ml. of ATP (1·2 μmoles), 0·1 ml. of sodium fumarate (10 μmoles), 0·1 ml. of sodium fluoroacetate (10 μmoles), and 1% KCl to total volume of 3·0 ml. Three flasks had an addition of 0·1 ml. of sodium pyruvate (30 μmoles).

	Citrate formed (μmole/flask)
A. No pyruvate	0·18
B. + pyruvate	0·81

The contents of flasks *A* and *B* were heated in thin-walled tubes in the boiling-water baths for 10 min. The supernatant fluid after cooling and centrifuging was used for the second experiment.

Second stage. The brain particle dispersion was made up in 18 ml. of KCl–phosphate mixture. Other details were the same, except that all the flasks contained fumarate and pyruvate, and that 8 flasks were used (4 sets of duplicates).

	Citrate formed (μmoles/flask)
No addition	0·54
+ *A* (1/10 of flask contents)	0·59*
+ *B* (1/10 of flask contents)	0·50*
+ fluorocitrate (synthetic) 5γ	1·31

Experiment 2. Failure of addition of CoA to influence the synthesis

In a similar set of two experiments carried out with the addition of CoA, there was also no appreciable citrate formation, even though the preparation was more active.

		Citrate formed (μmoles/flask)
First stage:	*A*. No pyruvate + CoA (36 units)	0·18
	B. + pyruvate + CoA (36 units)	0·64
Second stage:	No addition	0·99
	+ *A*	0·99*
	+ *B*	0·87*
	+ fluorocitrate	2·46

* Citrate present in 1/10 of *A* and *B* has been subtracted.

excluded. There can be no doubt therefore that in the kidney-tissue homogenate acetate stops fluorocitric acid formation. It is curious that on the whole there seems little correlation between O_2 uptake and the citric acid metabolism, but this has been also a feature of our earlier work on the fluorine-containing compounds.

Our biochemical results *in vitro* provide the biochemical proof in cell-free preparations that acetate is a powerful protective agent, in the sense that it stops the formation of fluorocitric acid. *In vivo*, as mentioned above, the effective behaviour of acetic acid itself has proved variable; except in mice (Tourtellotte & Coon, 1949; 1951) it works poorly. In the intact animal it appears therefore that acetic acid does not always reach the active centres concerned.

Though acetate has been here proved by two independent methods of estimation to inhibit fluorocitrate synthesis from fluoroacetate, the centre concerned with fluoroacetate activation is not on the ordinary pathway for acetate, because it has been a general experience that the known isolated acetate-activating systems do not activate fluoroacetate. For instance, some time ago we tried the acetone-dried pigeon-liver preparation (Stern & Ochoa, 1949). In two experiments with liver enzyme which easily formed citrate from oxaloacetate, in the presence of ATP, Mg^{2+} ions and cysteine, there was no detectable formation of fluorocitrate from oxaloacetate and fluoroacetate. Similar negative results have been obtained by us with acetate-activating enzymes from bacteria, kindly given to us by Dr W. H. Elliott. Others have had like experiences, for instance P. Hele (personal communication), Brady (1955), Dominguez, Shideman, Mahler & Hift (1954). Since it seems unlikely that acetate is merely blocking a centre through which it is not metabolized at all, it can be concluded that some stage of the pathway for the activation of fluoroacetate is independent of the usual main path for acetate activation; the final stage, however, appears to be fluoroacetyl-CoA as shown by Brady (1955) and Marcus & Elliott (1956). Hence the acetate inhibition of fluorocitrate synthesis appears to occur by some subsidiary pathway for acetate (Peters, 1955). It has been found by others (Hendershot & Chenoweth, 1954) that butyrate does not protect against fluoroacetate, but protects against fluorobutyrate. In a few experiments we have found that butyrate does not stop fluorocitric acid formation from fluoroacetate with the kidney particles, even at a concentration of 20 μmoles/3·0 ml. Hence, though the final step again should be fluoroacetyl-CoA, the inhibition by fluoro derivatives of higher fatty acids cannot occur on the same initial enzyme centre as that which activates the fluoroacetate; the position has been represented diagrammatically by Peters (1957). One further point may be mentioned: the experiment given in Table 1 confirms that the substances taking part in fluorocitrate synthesis belong to the

Table 5. *Effect of acetate on the synthesis of fluorocitric acid with kidney particles as tested by experiments with brain particles*

First stage. Kidney particles from guinea pig, prepared in KCl–phosphate mixture (see Table 3). Each flask contained 1·9 ml. of enzyme, 0·1 ml. of $MgCl_2$ (4·0 μmoles), 0·1 ml. of ATP (1·2 μmoles), additions as stated (0·2 ml. of malate (20 μmoles), 0·1 ml. of fluoroacetate (20 μmoles), 0·05–0·2 ml. of sodium acetate and KCl to bring to volume of 3·0 ml.). The flasks were shaken for 50 min. at 38°, cooled and immersed separately in thin test tubes in the boiling-water bath for 5 min. The supernatant was used for tests in the second experiment after centrifuging; it was stored in the cold.

Additions	Citrate accumulated (μmoles/flask)
A. Malate alone	0·53
B. Malate + fluoroacetate	5·80
C. Malate + fluoroacetate + sodium acetate (0·625 μmole)	5·10
D. Malate + fluoroacetate + sodium acetate (1·25 μmoles)	5·13
E. Malate + fluoroacetate + sodium acetate (2·50 μmoles)	6·40

Second stage. Brain-particle homogenate from two pigeons, prepared in KCl–phosphate mixture (not centrifuged) made up to 26·0 ml. Each respirometer flask contained, in a total volume of 3·0 ml., 1·9 ml. of enzyme, 0·1 ml. of $MgCl_2$ (4·0 μmoles), 0·1 ml. of ATP (1·2 μmoles), 0·2 ml. of pyruvate (30 μmoles), 0·1 ml. of fumarate (10 μmoles), 0·4 ml. of KCl–phosphate and additions (0·2 ml.) (i.e. 1/15 flask from 1st experiment). The flasks were shaken for 40 min. at 38°, and subsequently deproteinized with trichloroacetic acid for citric acid estimation. The effect is very marked with *D* (1·25 μmoles of acetate).

Additions	O_2 uptake (μl.)	Citrate accumulation (μmoles)
A	346	0·83
B	229	3·08
C	267	1·46
D	301	0·90
E	311	0·60

part of the tricarboxylic acid cycle not blocked by malonate, i.e. coming after succinate.

From the information available in this paper, in explaining fluoroacetate toxicity it is still unnecessary to go outside the hypothesis that fluorocitric acid is the toxic agent. On this hypothesis, the effect of the protective agents, monoacetin or acetamide when given early enough, is to stop the formation of fluorocitric acid *in vivo*, and so stop the poisoning.

In regard to the problem of acetate and fluoroacetate activation in brain tissue, there are some facts which are not yet reconciled: (1) No increase of oxygen uptake on adding acetate to brain-particle or -brei preparations has ever been found, i.e. these preparations do not activate acetate. (2) Acetate used with acetone-dried preparations of brain tissue can take part in the synthesis of acetylcholine via acetyl-CoA (Balfour & Hebb, 1952; Gardiner & Whittaker, 1954). (3) Fluoroacetate does not cause citrate accumulation *in vitro* in pigeon-brain tissue, which therefore lacks some of the enzymes needed for synthesis of fluorocitric acid. (4) Fluoroacetate when injected into the brain cortex of the dog *in vivo* does cause citric acid accumulation (Hendershot & Chenoweth, 1955). (5) Fluoroacetate when injected intraperitoneally or systemically into several species of animal causes convulsions with rise of citrate in brain tissue; this means that somehow fluorocitrate does get into the brain. (6) Fluorocitrate when injected into the subarachnoid space of pigeons, rats and rabbits induces convulsions at a much smaller concentration than fluoroacetate; in fact it is doubtful whether the latter produces convulsions directly at all (Hastings, Peters & Wakelin, 1955).

From (5) and (6) it seems certain that fluorocitrate is the actual initial stimulus to the convulsions; (4) has not actually been tried for dog's brain tissue *in vitro*. Points (3) to (6) would be reconciled if the tissue constituting the blood–brain barrier (whether blood vessel or otherwise) could synthesize fluorocitrate from fluoroacetate; the discrepancy between (1) and (2) may be merely a matter of relative concentration or of spatial separation.

SUMMARY

1. The effect of acetate *in vitro* on the synthesis of fluorocitric acid from fluoroacetate by particulate preparations from the guinea-pig kidney has been examined. Estimation of fluorocitric acid formed was made by two independent enzymic methods, with kidney and brain 'particles' respectively.

2. Both methods are in agreement in establishing that the presence of acetate in much lower concentrations than that of the fluoroacetate inhibits the synthesis of fluorocitric acid.

3. Since there is evidence that the usual acetate-activating systems do not activate fluoroacetate, the activation concerned here must take place on some independent centre.

4. In animals that are not protected by acetate, this substance must fail to penetrate to the active centres *in vivo*.

REFERENCES

Balfour, W. E. & Hebb, C. (1952). *J. Physiol.* **118**, 94.
Bartlett, G. R. & Barron, E. S. G. (1947). *J. biol. Chem.* **170**, 67.
Brady, R. O. (1955). *J. biol. Chem.* **217**, 213.
Buffa, P. & Peters, R. A. (1949). *J. Physiol.* **110**, 488.
Chenoweth, M. B., Kandel, A., Johnson, L. B. & Bennett, D. R. (1951). *J. Pharmacol.* **102**, 31.
Dominguez, A. M., Shideman, F. E., Mahler, H. R. & Hift, H. (1954). *Fed. Proc.* **13**, 1149.
Farah, A., West, T. C. & Angel, R. (1950). *J. Pharmacol.* **98**, 234.
Gardiner, J. F. & Whittaker, V. P. (1954). *Biochem. J.* **58**, 24.
Gitter, S. (1956). *Biochem. J.* **63**, 182.
Gitter, S., Blank, I. & Bergman, E. D. (1953). *Proc. kon. ned. Akad. Wet.* **56**, 4C, 423, 427.
Hastings, A. B., Peters, R. A. & Wakelin, R. W. (1955). *Quart. J. exp. Physiol.* **40**, 258.
Hendershot, L. C. & Chenoweth, M. B. (1954). *J. Pharmacol.* **110**, 344.
Hendershot, L. C. & Chenoweth, M. B. (1955). *J. Pharmacol.* **113**, 160.
Hutchens, J. O., Wagner, H., Podolsky, B. & McMahon, T. M. (1949). *J. Pharmacol.* **95**, 62.
Liébecq, C. & Peters, R. A. (1949). *Biochim. biophys. Acta*, **3**, 215.
Lotspeich, W. D., Peters, R. A. & Wilson, T. H. (1952). *Biochem. J.* **51**, 20.
Marcus, A. & Elliott, W. B. (1956). *J. biol. Chem.* **218**, 823.
Martius, C. (1949). *Liebigs Ann.* **561**, 227.
Morrison, J. F. & Peters, R. A. (1954). *Biochem. J.* **58**, 473.
Peters, R. A. (1952). *Brit. med. J.* **2**, 1165.
Peters, R. A. (1955). *Johns Hopk. Hosp. Bull.* **97**, 1.
Peters, R. A. (1957). *Advanc. Enzymol.* (in the Press).
Peters, R. A. & Wakelin, R. W. (1953). *J. Physiol.* **119**, 421.
Peters, R. A., Wakelin, R. W., Buffa, P. & Thomas, L. C. (1953). *Proc. Roy. Soc.* B, **140**, 497.
Peters, R. A. & Wilson, T. H. (1952). *Biochim. biophys. Acta*, **9**, 310.
Pucher, G. W., Sherman, C. C. & Vickery, H. B. (1936). *J. biol. Chem.* **113**, 235.
Rivett, D. E. A. (1953). *J. chem. Soc.* p. 3710.
Stern, J. R. & Ochoa, S. (1949). *J. biol. Chem.* **179**, 491.
Tourtellotte, W. W. & Coon, J. M. (1949). *Fed. Proc.* **8**, 339.
Tourtellotte, W. W. & Coon, J. M. (1951). *J. Pharmacol.* **101**, 82.

The Formation of Glycine and Serine

THE INFLUENCE OF THE ADMINISTRATION OF GLYCINE, DL-SERINE AND OTHER COMPOUNDS ON LEVELS OF TISSUE GLYCINE AND SERINE

BY J. L. SIMKIN* AND K. WHITE
Department of Physiology, The Medical School, University of Birmingham

(*Received* 8 *March* 1957)

The rates of conversion of glycine into serine and serine into glycine have been determined by administration of isotopically labelled glycine and serine (Arnstein & Neuberger, 1953; Elwyn & Sprinson, 1954; Arnstein & Keglević, 1956; Arnstein & Stanković, 1956). The present investigation, which is concerned with the net synthesis of glycine from serine, and of serine from glycine, was carried out with unlabelled glycine and serine and the methods were similar to those used in a previous investigation of tissue-glycine levels (Simkin & White, 1957). Greenberg & Sassenrath (1955) have made a more extensive but less detailed investigation of the effect of administration of several amino acids including glycine and serine.

METHODS

Animals, diet and method of dosage were described by Simkin & White (1957). The dose level of amino acids and of sodium benzoate was usually about 400 μmoles/100 g. body wt. In the rat the volume injected was generally 1·0 ml., except with DL-serine where its low solubility made it necessary to use 1·5–2·0 ml. If several compounds were administered to the same animal they were, if possible, dissolved in the same solution, but where this was not possible the total volume injected did not exceed 2·0 ml. Control rats were injected with an equal volume of water. Rabbits were usually given 10 ml. (20 ml. for DL-serine). Pyruvic acid was neutralized with $NaHCO_3$ before dilution to the required volume.

Collection and deproteinization of tissues

Blood. This has been described previously (Simkin & White, 1957).

Other tissues. The method of Simkin & White (1957) was used when only glycine was determined, but this was modified, as suggested by Schurr, Thompson, Henderson & Elvehjem (1950), when filtrates were prepared for the estimation of serine by microbiological assay, in the following way. The frozen sample (3–4 g.) was heated with 10 ml. of water in a boiling-water bath for 3 min. The cooled sample was comminuted with a further 10 ml. of water and the requisite amount of tungstate mixture. Tungstic acid filtrates were stored under toluene in a refrigerator overnight and brought to pH 6·8–7·0 before assay.

Determination of serine

Serine was estimated by microbiological assay, with *Leuconostoc mesenteroides* P60 and a slight modification of the method of Henderson & Snell (1948). Sodium benzoate (final concn. 0·1 mM) did not interfere with the growth of the organism (cf. Nurmikko & Virtanen, 1951). Krueger (1950) used a similar method of assay and reported that β-hydroxybutyric and pyruvic acids had no effect upon the growth rate of this organism.

Plan of experiments

This was, in general, as described by Simkin & White (1957). The mean control value obtained from all experiments was used for rats. Microbiologically available serine has been termed 'free L-serine'.

RESULTS

The results obtained for the levels of glycine and serine in rat liver and blood and in rabbit blood are presented in Tables 1–3. Table 1 contains values obtained in rat liver after administration of glycine, Table 2 those after administration of serine and its precursors, and Table 3 those for levels of glycine and serine in rat and rabbit blood.

Administration of glycine

The intraperitoneal injection of glycine caused a rapid rise in the concentration of free glycine in rat liver (Table 1). The concentration of free L-serine in liver was similarly affected. The ratio of the concentration of serine to that of glycine remained approximately constant. The control value of this ratio was 0·45, and the mean value after administration of glycine was 0·46 (± S.D. 0·14, 8 values).

Table 3 shows the results of experiments on the effect of injection of glycine into the rat and rabbit. The blood glycine level increased about 12-fold at 0·5 hr. after injection. The concentration of free glycine in rat liver and in rat and rabbit blood increased also after simultaneous injection of equimolecular quantities of glycine and benzoate (Tables 2 and 3), but the increase was smaller than that observed when a similar dose of glycine was administered alone (Table 1). [A similar result was obtained by de Vries & Alexander (1948), who

* Present address: National Institute for Medical Research, Mill Hill, London, N.W. 7.

studied the free glycine concentration of human plasma.] Although the rise in glycine concentration observed was smaller than when glycine alone was administered, the concentration of free serine in rat liver still increased (Table 2).

Administration of serine and serine precursors

The concentration of free serine in the liver was raised to a considerable extent by administration of DL-serine, acetate, pyruvate and DL-β-hydroxybutyrate (Table 2). There was not a marked rise in the level of glycine. The ratios of the concentrations of serine to those of glycine were well above the control value (0·45) and ranged from a minimum of 0·75 after the administration of pyruvate to 1·40 after administration of DL-serine.

The concentration of serine, after administration of DL-serine + benzoate, was raised to approximately the same extent as in the absence of benzoate, and the administration of serine failed to prevent the decrease in glycine concentration normally found in the presence of benzoate (Simkin & White, 1957); the ratio of serine to glycine concentration was about 2·8.

After administration of glycine and benzoate the concentrations of both free L-serine and glycine in

Table 1. *Effect of intraperitoneal injection of glycine on the concentration of free glycine and serine in rat liver*

Each determination was carried out on the pooled livers from two rats. Mean control values for undosed animals were: free glycine, 268±42 S.D. (33 expts.); free (microbiologically available) L-serine, 120±9 S.D. (7 expts.). Column headings in hr. refer to time of death; doses were injected at 0 hr.

Expt.	Dose (μmoles/100 g. body wt.)	Free glycine (μmoles/100 g. of fresh tissue) 0·5 hr.	1·5 hr.	2·0 hr.	3·0 hr.	5·0 hr.	Free L-serine (μmoles/100 g. of fresh tissue) 0·5 hr.	1·5 hr.	3·0 hr.	5·0 hr.
A	360	980	—	530	—	—	—	—	—	—
B	190	655	—	355	—	—	—	—	—	—
C	380	835	535	—	525	420	—	—	—	—
D	460	1250	770	—	405	255	365	335	170	105
E	390	950	515	—	345	—	455	275	265	—
F	430	1160	—	—	—	—	410	—	—	—

Table 2. *Effect of intraperitoneal injection of serine and serine precursors on the concentration of free glycine and serine in rat liver*

Each determination was carried out on the pooled livers from two rats. Mean control values for undosed animals: free glycine, 268±42 S.D. (33 expts.); free (microbiologically available) L-serine, 120±9 (7 expts.). Column headings in hr. refer to time of death; doses were injected at 0 hr.

Expt.	Compound administered	Dose (μmoles/100 g. body wt.) Serine or precursor	Benzoate	Free glycine (μmoles/100 g. of fresh tissue) 0·25 hr.	0·5 hr.	1·5 hr.	3·0 hr.	5·0 hr.	Free L-serine (μmoles/100 g. of fresh tissue) 0·25 hr.	0·5 hr.	1·5 hr.	3·0 hr.	5·0 hr.
G	DL-Serine	380	—	—	260	300*	—	—	—	—	—	—	—
H		410	—	300†	245‡	—	—	—	—	—	—	—	—
I		1000§	—	—	310	280‖	—	—	—	—	—	—	—
J		400	—	—	320	315	305	265	—	—	—	—	—
K		440	—	—	300	270	255	—	—	415	295	260	—
L	DL-Serine +	420	420	—	140	185	115	105	—	—	—	—	—
M	benzoate	410	380	—	145	115	90	—	—	430	295	250	—
N	Acetate	400	—	180	—	—	—	—	195	—	—	—	—
O		1000	—	—	240	—	—	—	—	265	—	—	—
P	Pyruvate	400	—	215	—	—	—	—	165	—	—	—	—
Q		1000	—	—	210	—	—	—	—	190	—	—	—
R	DL-β-Hydroxy-	400	—	260	—	—	—	—	230	—	—	—	—
S	butyrate	1000	—	—	270	—	—	—	—	260	—	—	—
T	Glycine +	350	350	—	730	405	200	240	—	—	—	—	—
U	benzoate	400	370	—	605	260	110	—	—	375	255	190	—
V	Benzoate	—	460	—	90	125	100	105	—	90	125	95	150
W		—	470	—	—	115¶	170	90	—	—	120¶	165	75

* 2 hr. † 0·35 hr. ‡ 0·75 hr.
§ Owing to the sparing solubility of DL-serine, 600 μmoles/100 g. of this dose were given by stomach tube.
‖ 0·9 hr. ¶ 1 hr.

Table 3. *Effect of intraperitoneal injection of serine, glycine and benzoate on the concentration of free glycine and serine in blood*

Each determination for rats was carried out on the pooled blood of two animals. Mean control values for undosed animals were: free glycine 20±4 S.D. (29 expts.); free (microbiologically available) L-serine 22±8 (4 expts.). For rabbits, blood samples were withdrawn at the stated times and the control value (0 hr.) for each animal is given. Column headings in hr. refer to time of death of rats, or time of taking sample from rabbits; doses were injected at 0 hr. Lettered experiments refer to rats and numbered experiments to rabbits.

Expt.	Compound administered	Dose (μmoles/100 g. body wt.) Glycine or serine	Benzoate	Free glycine (μmoles/100 g.) 0 hr.	0·25 hr.	0·5 hr.	1·5 hr.	3 hr.	5 hr.
C	Glycine	380	—	—	—	260	106	52	44
T	Glycine + benzoate	350	350	—	—	114	52	18	23
J	DL-Serine	400	—	—	—	19	18	17	17
L	DL-Serine + benzoate	420	420	—	—	10	12	9	11
1	Glycine	430	—	63	720	860	820	530	310
2	Glycine + benzoate	390	390	57	210	250	187	120	69
3	DL-Serine	300	—	167	165*	157	151	162	143
4	DL-Serine + benzoate	290	290	107	84*	74	47	27	16
				Free L-serine (μmoles/100 g.)					
V	Benzoate	—	460	—	—	20	16	17	16
W	Benzoate	—	470	—	—	10†	—	16	12
5	Benzoate	—	400	33	—	36‡	29	30	—

* 0·3 hr. † 1 hr. ‡ 0·75 hr.

the liver were increased initially above the control value in almost the same proportion, but subsequently the serine level decreased more slowly than the glycine level (Table 2). The ratio of serine to glycine concentration rose from 0·62 to 1·9. Administration of DL-serine did not result in any significant change in the concentration of free glycine in rat or rabbit blood (Table 3).

Administration of benzoate

The concentrations of free glycine and L-serine in rat liver after administration of benzoate are given in Table 2. There is no evidence for a large change in concentration of free serine, but since the variation (S.D. ± 32) appears to be greater than that in the controls (S.D. ± 9) no conclusion can be drawn as to the possibility of a small variation. The concentration of free glycine in the same experiments decreased, as is usual after benzoate administration (Simkin & White, 1957). The change in the concentration of free serine in the blood of a rabbit after the administration of benzoate was small (Table 3). Injection of serine with benzoate did not prevent the decrease in free glycine of the blood, which is characteristic of benzoate administration.

DISCUSSION

Formation of serine from glycine

The change in tissue levels of serine after administration of glycine show that there is a rapid net synthesis of serine from glycine, which indicates that the rate of formation of serine from glycine varies markedly with the tissue level of glycine. It had already been shown (e.g. by Arnstein & Neuberger, 1953) that glycine may provide both the hydroxymethyl group of serine and the remaining part, but investigations with labelled glycine did not exclude the possibility that the rate of the reaction was limited by a reaction step following zero-order kinetics. It is clear that this is not so, and that glycine from dietary sources can act as an effective precursor of serine.

This fact is of considerable interest in connexion with the glycogenic action of glycine. Although Hess (1950) found that glycogen was rapidly formed from administered DL-serine, there is a time lag of several hours before glycogen is formed from administered glycine (see Arnstein, 1954), and it has been suggested (Arnstein, 1954) that a possible explanation of this lag is the limited net rate of conversion of glycine into serine. The results obtained from the present investigation do not support this explanation.

Formation of glycine from serine

It is known that glycine is readily formed from serine, since administration of isotopically labelled serine results in the production of glycine with a specific activity as high as 63% of that of the administered serine (Elwyn & Sprinson, 1954). We have found, however, that the tissue levels of glycine after administration of serine showed little or no increase above normal values, which indicates that the rate of conversion of serine into glycine varies little with the tissue level of serine. An

increase in the tissue level of serine resulting from administration of nitrogen-free precursors also fails to change the tissue level of glycine, showing that the lack of effect of administered serine is not caused by permeability barriers. Moreover, when serine is formed from administered glycine, and the level of glycine is reduced by benzoate, the serine level decreases more slowly than that of glycine.

The present investigation shows that there is no evidence for an increase in the level of free glycine in liver after administration of serine or its precursors (see, however, Christensen, Streicher & Elbinger, 1948; Greenberg & Sassenrath, 1955). The mean of 19 values after administration of DL-serine, acetate, pyruvate or DL-β-hydroxybutyrate was 264 ± 37 S.D.; the mean of 33 control values was 268 ± 42. The fact that the value of the standard deviation of the mean free glycine level of liver after administration of serine or its precursors is very close to that of the control confirms that this calculation of the mean value of the determinations at different time intervals after dosage is permissible; calculation of such an average value would tend to obscure a temporary rise in the free glycine level of liver, but such a rise would lead to a large standard deviation. The mean of 12 values for free serine in liver was 270 ± 81 as compared with a control value of 120 ± 9 (7 expts.). The standard deviation of 81 corresponds to real differences in the effects of administering serine or its precursors, as well as to temporary changes in the level of free L-serine. The evidence shows that an increase in the tissue level of serine has much less effect on the conversion of serine into glycine than an increase in the tissue level of glycine has on the formation of serine. This may be expressed formally by saying that the kinetics followed by the serine $\rightarrow$ glycine reaction approach zero- rather than first-order. This is supported by the inability of DL-serine to restore the growth rate of glycine-depleted rats (White, 1941).

Availability of serine and glycine

The results of Arnstein & Neuberger (1953) show that the gross rate of formation of glycine from serine and other precursors is independent of the supply of glycine in the diet. As shown in the previous section, this rate is only slightly increased by the administration of serine. The present investigation shows that there is a net formation of serine from glycine after administration of glycine, which indicates that the gross rate of formation of serine increases with increasing glycine level.

These findings seem to suggest that in the absence of dietary sources of glycine the net production of glycine from serine will increase with the requirements of the organism for glycine. As the requirement for glycine increases, the tissue level of glycine will decrease. An extreme instance of this is the marked decrease in free glycine of the liver after administration of benzoate (Table 2). The rate at which glycine is converted into serine will therefore decrease, so that if the serine level stays constant the net production of glycine would be greater than before. The present results suggest, however, that the reduction in the glycine level after administration of benzoate does not affect the rate of formation of serine. It is known that the α-C atom of glycine is a source of the β-C atom of serine, presumably via formaldehyde or a derivative at the same level of oxidation (Elwyn & Sprinson, 1954). When there is a plentiful supply of glycine, up to 60% of the β-C atoms of serine is derived from glycine, but at normal glycine levels glycine acts as precursor of the β-C atom of serine to a negligible extent (Arnstein & Neuberger, 1953). Under these conditions, therefore, glycine is of little importance as a source of 'formaldehyde', and a lowering of the glycine level would not appreciably diminish the availability of 'formaldehyde'. The fact that serine levels are maintained even when the glycine level is depressed by benzoate suggests that the rate of formation of serine is limited by the concentration of 'formaldehyde' rather than of glycine.

SUMMARY

1. The influence of the administration of benzoate, glycine, DL-serine, acetate, pyruvate or DL-β-hydroxybutyrate on the concentrations of free glycine and free L-serine in the liver and blood of the rat and the blood of the rabbit has been investigated. Administration of glycine resulted in a rise in the concentration of both glycine and serine. Administration of serine or its non-nitrogenous precursors resulted in a rise in the concentration of serine but not of glycine. Administration of benzoate alone resulted in a fall in the concentration of glycine but not of serine.

2. The significance of the results is discussed with reference to the kinetics of the interconversion of glycine and serine.

We wish to thank Dr F. W. Norris for providing a culture of *Leuconostoc mesenteroides*. We are indebted to Dr W. V. Thorpe and Dr H. G. Bray for their interest and discussion of this work and to Mr P. B. Wood, Mrs B. G. Taylor and Miss D. A. Clarke for technical assistance.

REFERENCES

Arnstein, H. R. V. (1954). *Advanc. Protein Chem.* **9**, 80.
Arnstein, H. R. V. & Keglević, D. (1956). *Biochem. J.* **62**, 199.
Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 271.
Arnstein, H. R. V. & Stanković, V. (1956). *Biochem. J.* **62**, 190.

Christensen, H. N., Streicher, J. A. & Elbinger, R. L. (1948). *J. biol. Chem.* **172**, 515.
de Vries, A. & Alexander, B. (1948). *J. clin. Invest.* **27**, 665.
Elwyn, D. & Sprinson, D. B. (1954). *J. biol. Chem.* **207**, 459.
Greenberg, D. M. & Sassenrath, E. N. (1955). *Cancer Res.* **15**, 620.
Henderson, L. M. & Snell, E. E. (1948). *J. biol. Chem.* **172**, 15.
Hess, W. C. (1950). *J. Amer. chem. Soc.* **72**, 1407.
Krueger, R. (1950). *Helv. chim. acta*, **33**, 2157.
Nurmikko, V. & Virtanen, A. I. (1951). *Acta chem. scand.* **5**, 97.
Schurr, P. E., Thompson, H. T., Henderson, L. M. & Elvehjem, C. A. (1950). *J. biol. Chem.* **182**, 29.
Simkin, J. L. & White, K. (1957). *Biochem. J.* **65**, 574.
White, A. (1941). *Yale J. biol. Med.* **13**, 759.

Determination of Manganese in Tissue extracts by means of Formaldoxime

By W. BARTLEY, BRENDA M. NOTTON and W. C. WERKHEISER*
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

(*Received* 20 *March* 1957)

Sideris (1937) described a method for the estimation of Mn^{2+} ions in ashed tissue which was based on the spot-test reaction of manganese with formaldoxime (CH_2:N·OH). Gum ghatti was used to stabilize the colour. The present modification of this method was developed for the study of the uptake of Mn^{2+} ions by tissues from dilute solutions (10^{-3} to 10^{-5} M). The estimation is made directly on trichloroacetic acid or perchloric acid extracts of the tissue without prior ashing. The technique is simple, involving the addition of a single solution, and the colour developed is extremely stable without addition of any protective colloid. Ferric iron which also reacts with formaldoxime may be removed by extraction as ferric thiocyanate. Between 0·01 and 0·25 μmole (0·55–13·7 μg.) of manganese can be estimated in this way. Yellow tissue constituents absorb at the same wavelength as the formaldoxime–manganese complex and this may reduce the accuracy of the determination at low levels of Mn^{2+} ions. The method is therefore not suitable for the determination of endogenous tissue manganese without prior ashing, but it is very convenient for the determination of manganese at the levels often used in enzyme experiments.

METHOD

Solutions. Manganous chloride (A.R., 0·1 M) in glass-distilled water was diluted to give a working standard of 0·0005 M.

A solution of formaldoxime hydrochloride (British Drug Houses Ltd.), 0·5 g. in 10 ml. of glass-distilled water, was made; 0·1 ml. of this solution was diluted with 1 ml. of 10 N-NaOH immediately before use.

* Present address: Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.

Procedure

Tissue samples (liver homogenates or mitochondrial suspensions) were deproteinized with trichloroacetic acid (final concn. 0·8 N) or $HClO_4$ (final concn. 0·5 N). The clear supernatant obtained after centrifuging constitutes the tissue extracts which were analysed. Up to 3·0 ml. of the tissue extract was placed in a test tube. With the smaller samples the volume was adjusted to 3·0 ml. with water. To ensure rapid mixing 1 ml. of formaldoxime was squirted from a 1 ml. all-glass hypodermic syringe into the test solution; mixing was completed at once by shaking the tube. Less accurate results were obtained when the mixing was not rapid. After 10 min. the optical density was measured in 1 cm. cells in the Unicam spectrophotometer SP. 600 at 450 mμ (see Fig. 1). The solutions were read against a manganese-free solution treated in the same way. The optical density of the blank solution increases slightly for the first 2 hr. and then steadily fades. However, in the test

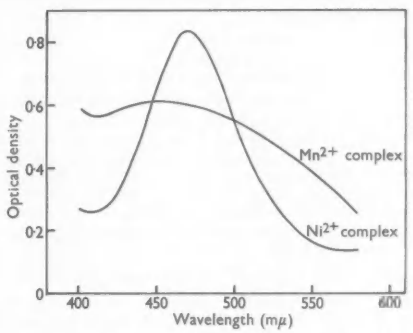


Fig. 1. Absorption spectra of manganese and nickel complexes with formaldoxime. Solutions (3 ml.) containing 0·25 μmole of Mn^{2+} or Ni^{2+} were treated with 1 ml. of formaldoxime solution.

solutions the colour of the manganese complex does not fade, i.e. the difference between optical density of the test and that of the blank does not vary with time. The coloured manganese complex is stable for at least 5 days. Known amounts of manganese were carried through the procedure with each set of estimations to construct a standard curve. This was linear over the range 0–0·25 μmole of Mn^{2+} and 0·25 μmole of Mn^{2+} gave an optical density of approximately 0·56 at 450 mμ. Duplicate determinations agreed within 2% and therefore only single measurements were made as a rule.

RESULTS

Interfering substances

Iron. With Fe^{3+} ions formaldoxime forms a purple complex which develops much more slowly than the colour of the manganese and reaches its maximum in about 2–3 hr. The absorption when measured at 450 mμ is about half that of a manganese solution of the same concentration. Sideris (1937) recommends the addition of cyanide to overcome the interference of the Fe^{3+} ions. This was unsatisfactory under our conditions. Fe^{3+} ions in the presence of excess of ammonium thiocyanate were extractable from water solutions into ethyl acetate, whereas Mn^{2+} ions remain in the aqueous phase. This offered a possible method for the removal of iron from manganese solutions before the addition of the formaldoxime reagent.

The sample of tissue extract containing not more than 2·5 μmoles of Fe^{3+} and 0·5 μmole of Mn^{2+} was placed in a 100 ml. separating funnel, or more conveniently in the extracting funnels described by Bartley (1953). To the sample was added 0·1 ml. of 10% aqueous ammonium thiocyanate solution, 0·1 ml. of N-H_2SO_4, water to 3 ml. and 10 ml. of ethyl acetate. The mixture was shaken for about 30 sec., and after a few seconds the lower aqueous layer was run into a second funnel containing 10 ml. of ethyl acetate and the extraction procedure was repeated. The aqueous layer was run off into a graduated 10 ml. tube. Water (2 ml.) was added to the first funnel and after extraction the aqueous layer was run into the second funnel. After re-extraction the aqueous layer was added to the contents of the graduated tube. The volume was adjusted to 6 ml. with water. To a 3 ml. sample of this solution was added 1 ml. of the formaldoxime reagent and the mixture was left for 24 hr. before reading. This long delay was to allow the purple colour that developed on the addition of the reagent following the extraction procedure to fade. To test the procedure tissue extracts containing known amounts of Mn^{2+} or Mn^{2+} and Fe^{3+} ions were analysed.

In Table 1 (*A*) are given the values obtained by the direct method when no Fe^{3+} ions were added, and in Table 1 (*B*) are given comparable values after extraction of the added Fe^{3+} ions.

Nickel. Ni^{2+} ions interfere with the determination as they produce a yellow–brown colour with formaldoxime with about the same molar extinction coefficient as the Mn^{2+} complex at 450 mμ. It is possible that the difference in the two absorption spectra (Fig. 1) might give a basis for the measurement of manganese in the presence of nickel. This possibility has not been further investigated.

Copper. Vogel (1948) states that Cu^{2+} ions give a blue–violet colour with formaldoxime. Under our test conditions when the amount of Cu^{2+} ions added did not exceed 0·25 μmole the colour was transient and did not interfere with the determination of Mn^{2+} ions. With larger amounts of copper the blue colour was more permanent but could be removed by the addition of a small amount of solid sodium cyanide. As shown below NaCN does not interfere with the determination of Mn^{2+} ions.

Chromium and cobalt. Vogel (1948) also states that Cr^{3+} and Co^{2+} ions give colours with formaldoxime. The colour with chromium was found to be negligible and that with cobalt was less than 5% of the Mn^{2+} complex at the same concentration. Moreover, the cobalt complex was extracted by the procedure used for the removal of iron.

Calcium. At the alkaline pH required for the formation of the formaldoxime complex calcium salts form a precipitate. Removal of the precipitate by centrifuging results in a clear supernatant with the same optical density as a similar solution without the addition of calcium.

Phosphates. Sideris (1937) has stated that phosphate interfered with his method. At levels above

Table 1. *Manganese determinations by direct and extraction methods*

Manganese was measured either directly or after treatment with KSCN ethyl acetate as described in the text. Test samples (values *A*) were tissue extracts containing the amounts of Mn^{2+} ions given in column 1. To each of a further similar set of samples was added 2·5 μmoles of Fe^{3+} ions. These were analysed by the extraction procedure and gave the values *B*.

Added Mn^{2+} ion content (μmole/3 ml.)	Optical density by standard method (*A*)	Optical density after extraction procedure (*B*)	100*B*/*A*
0·025	0·065	0·066	101
0·050	0·123	0·127	103
0·10	0·330	0·324	98
0·150	0·400	0·386	96
0·25	0·660	0·640	97

Table 2. *Effect of phosphates on the estimation of manganese by formaldoxime*

The amount of phosphate indicated was added to the 3 ml. of the test sample.

	Optical density			
Phosphate added (μmoles) ...	0	10	20	40
Mn^{2+} ions added (μmoles)				
0·05	0·140	0·146	0·123	0·131
0·10	0·284	0·278	0·264	0·271
0·25	0·682	0·652	0·665	0·692

40 μmoles in the test sample, phosphate also interferes in our method, but below this level no difference was found between the presence or absence of phosphate (Table 2).

Sucrose. As the fractionation of tissue homogenates is often carried out in sucrose solutions the recovery of manganese from sucrose containing suspensions was studied. Amounts of sucrose above 50 μmoles in the test sample decreased the colour formation. However, with any particular amount of sucrose the colour developed by the formaldoxime reagent was linear with respect to manganese concentration. The colour of the manganese–formaldoxime complex did not have the usual stability, some fading occurring after about 2 hr. After 24 hr. the colour had completely faded. Thus when sucrose is present in the test material at amounts greater than about 50 μmoles, the standard manganese solutions should also contain approximately the same amount of sucrose; in all cases when sucrose is present the optical density should be measured within 1 hr. of adding the formaldoxime.

If solutions containing both sucrose and Fe^{3+} ions were subjected to the extraction procedure the fading of the colour caused by sucrose did not occur. Presumably the extraction had removed or reduced this substance as well as Fe^{3+} ions to a level where interference did not occur.

Effect of metal-complex-forming agents. Many substances found in biological material or added in metabolic experiments have the property of 'binding' metal ions. The possible interference in the manganese method of some of the more common metal-complex-forming substances was therefore tested. None of the substances in the amounts given in Table 3 inhibited the reaction between manganese and formaldoxime. With cyanide the colour development was somewhat retarded, but after about 1 hr. the normal optical density was reached. The colour contribution of the cyanide solution was appreciable at the high levels. The reason for this colour is not clear but due allowance was made for it in calculating the percentage recoveries given in Table 3.

Recovery of manganese added to tissues. The recovery of manganese added to dilute mitochondrial suspensions (0·04–0·25 μmole of Mn^{2+} added to 2·0 ml. of suspension containing 40 mg. dry wt. of mitochondria) after deproteinization and extraction with 10 % trichloroacetic acid, ranged between 97 and 105 %. In similar tests with unfractionated liver homogenates in 0·9 % potassium chloride solution extracted by 10 % trichloroacetic acid, the recoveries were usually too high and ranged between 98 and 125 % recovery. When 0·5 N-perchloric acid was used as the extracting fluid the recoveries (102–112 %) were more consistent but were still too high. This excess of colour was due to the colour of the tissue extract being enhanced by Mn^{2+} ions. This enhancement occurred at low concentrations of Mn^{2+} ions (about 0·01 μmole in the 3 ml. of test sample) and did not increase with increase of the Mn^{2+} ions added. It was therefore of significance only when the Mn^{2+} ion content of the sample was low. Table 4 gives the recoveries of various amounts of Mn^{2+} ions when allowance was made for this correction.

Estimation of endogenous Mn^{2+} ions. As expected from the previous section the method could not be applied directly to the estimation of endogenous manganese in tissue extracts prepared in 0·5 N-$HClO_4$ or 5 % trichloroacetic acid. Table 5 shows a comparison between the apparent Mn^{2+} ion content of the tissue extract and the true Mn^{2+} ion content

Table 3. *Effect of metal-binding agents on the manganese–formaldoxime reaction*

The test solution contained 0·25 μmole of Mn^{2+}/3 ml.

Complex-forming agent added	Amount added (μmoles)	Ratio of optical densities with and without complex-forming agent × 100
Cysteine	30	100
ATP	4	97
	3	100
Cyanide	1250	99
	1000	97
	750	100
	500	99
Pyrophosphate	250	99
Ethylenediamine-tetra-acetic acid	700	102

Table 4. *Recovery of Mn^{2+} ions added to a liver homogenate*

Rat liver was homogenized in 9 vol. of 0·9 % KCl. Mn^{2+} ions were added to give in 0·5 ml. the amounts stated below. To the suspension was added 0·2 vol. of 2·5 N-$HClO_4$ and, after removal of the precipitated protein by centrifuging, 0·5 ml. of the extract was analysed for Mn^{2+} ions.

Addition to	Mn^{2+} ions added (μmole)	Mn^{2+} ions recovered (μmole)	Recovery (%)
$HClO_4$ extract of rat-liver homogenate	0·1	0·099	99
	0·15	0·151	101
	0·2	0·200	100
	0·25	0·252	101
Rat-liver homogenate kept 30 sec. then $HClO_4$ added	0·1	0·099	99
	0·15	0·155	103
	0·2	0·197	98
	0·25	0·25	100
Rat-liver homogenate kept 90 min. then $HClO_4$ added	0·1	0·100	100
	0·15	0·153	102
	0·2	0·197	99
	0·25	0·248	99

Table 5. *Manganese content of tissues*

The values given in the second column were calculated from the data of Skinner *et al.* (1931) for rats supplemented with 5 mg. of Mn^{2+} ions daily. The calculated Mn^{2+} ion intake of the rats used in the present work was 6–10 mg. daily. For other experimental details see text.

	Mn^{2+} ion content (μmole/g. of fresh tissue)		
	In ashed material		In $HClO_4$ extract.
Tissue	Skinner *et al.* (1931)	This paper	This paper
Rat liver	0·0373	0·047	0·064
Rat brain	0·0137	0·00967	0·017
Rat muscle	0·003	0·00646	0·0213
Sheep pancreas	—	0·0294	0·036
Pig pancreas	—	0·027	0·020

of the tissue extract after ashing by the method of Lorenzen & Smith (1947). A comparison is also given with the Mn^{2+} ion content of rat tissue as found by Skinner, Peterson & Steenbock (1931). These authors showed that the dietary intake of Mn^{2+} ions was reflected in the Mn^{2+} ion content of the liver and muscle. This may account for the somewhat higher values obtained with our rats, as their Mn^{2+} ion intake was about twice that of the Mn^{2+}-supplemented rats used by Skinner *et al.* (1931).

The manganese estimation gave the same value as the dry-ashed tissue if the perchloric or trichloroacetic acid extracts were submitted to a wet-ashing procedure [2·0 ml. of extract + 0·2 ml. of the ashing mixture of Hanes & Isherwood (1949) + 0·1 ml. of 5% ammonium molybdate solution boiled down to 0·2 ml.]. The wet-ashing procedure is somewhat less tedious than the dry-ashing of the whole tissue. However, it is clear that the formaldoxime method is not suitable for the routine analysis of tissues without prior ashing unless the Mn^{2+} ion content is relatively high. It is very well suited to the study *in vitro* of tissues supplemented with extra Mn^{2+} ions.

DISCUSSION

Manganese is usually estimated colorimetrically after oxidation to permanganate. The present method has the advantage over the permanganate method in that no removal of organic material is required, although it may be used on ashed material when this is necessary. No colour developed in the presence of phenols as occurs in the method of Abul-Fadl (1948). The estimation usually requires only the addition of a single solution and the great stability of the colour is also advantageous. A limitation on the method is imposed by the absorption at 450 mμ of yellow tissue constituents.

Ni^{2+} ions seriously interfere with the estimation and the method is not applicable in their presence. When the method is used on ashed tissue or tissue extracts the iron-extraction procedure must be used as the bound tissue-iron is liberated. In most trichloroacetic acid or perchloric acid extracts of tissues the free iron content is too small to interfere and the iron-extraction procedure is unnecessary. The presence of iron in the extract may be tested for by adding ammonium thiocyanate; if a definite red does not appear the iron extraction may be omitted. The presence of thiocyanate does not interfere with the subsequent manganese determination.

The method has the merit of being insensitive to the effect of chelating agents such as ethylenediaminetetra-acetic acid and cyanide, both of which are often added to tissue preparations. The reason for the inhibitory action of sucrose is not clear as this material is not usually considered a powerful chelating substance.

SUMMARY

1. The reaction of manganese with formaldoxime, first used by Sideris (1937) as the basis of the quantitative determination of manganese, has been modified to give a simple method for the estimation of manganese in perchloric or trichloroacetic acid extracts of biological material. From 0·01 to 0·25 μmole of Mn^{2+} in 3 ml. of tissue extract can be measured with less than 5% error. Yellow tissue constituents absorb at the same wavelength as the formaldoxime–manganese complex and reduce the accuracy of the determination when present in large amounts. The method is not suitable for the determination of endogenous tissue manganese without prior ashing, but it works well at levels (10^{-3} to 10^{-5} M) often used in enzyme experiments.

2. Iron and nickel react with formaldoxime and interfere with the estimation. The removal of iron by extraction is described.

3. The method can be used in the presence of large amounts of metal complex-forming agents such as cyanide or ethylenediaminetetra-acetic acid. Cyanide causes some retardation in the colour development and sucrose causes the colour to fade after 2 hr.

We wish to thank Professor H. A. Krebs, F.R.S., for his interest and advice in the work, and Mr Alan Renshaw for making the figures. One of us (W.C.W.) is a Research Fellow of the Natural Cancer Institute, U.S. Public Health Service. The work was aided by a grant from the Rockefeller Foundation.

REFERENCES

Abul-Fadl, M. A. M. (1948). *Biochem. J.* **42**, xxxvii.
Bartley, W. (1953). *Biochem. J.* **54**, 677.
Hanes, C. S. & Isherwood, F. S. (1949). *Nature, Lond.*, **164**, 1107.
Lorenzen, E. J. & Smith, S. E. (1947). *J. Nutr.* **33**, 143.
Sideris, C. P. (1937). *Industr. Engng Chem.* (*Anal.*), **9**, 445.
Skinner, J. T., Peterson, W. H. & Steenbock, H. (1931). *J. biol. Chem.* **90**, 65.
Vogel, A. I. (1948). *A Textbook of Qualitative Chemical Analysis*. London: Longmans, Green and Co.

Application of the Carbazole Reaction to the Estimation of Glucuronic Acid and Glucose in some acidic Polysaccharides and in Urine

By J. M. BOWNESS
Department of Biochemistry, University of Malaya, Singapore

(*Received* 18 *December* 1956)

A number of carbohydrates may be detected and estimated by means of a reaction with carbazole in sulphuric acid. The methods used have been summarized by Dische (1955). The present paper describes a procedure for the estimation of mixtures of glucuronic acid, glucose and xylose in pure solution, and of glucuronic acid and glucose in various polysaccharides and in urine. Work is also described indicating that the colours obtained with hexoses and pentoses are due to hydroxymethylfurfural and furfural respectively, and that the colour given by the uronic acids is due to neither of these compounds.

Dische (1947) observed that the addition of water to the coloured complex which is slowly formed after mixing carbazole and glucuronic acid in 87% sulphuric acid caused the colour to change and lighten, whereas the slight colour obtained with glucose and other sugars by the same process was intensified. In the present procedure one reading is taken with an absorptiometer 2·5 hr. after the reactants have been mixed; glucuronic acid gives an intense colour at this stage. Water is added, and two further readings are taken on the following day, one with a yellow–green and one with a violet filter. Glucose gives a more intense colour than glucuronic acid at this stage, with maximum absorption of light in the yellow–green region. Pentoses also give a colour at this stage with maximum absorption in the yellow–green region. The pentose colour is less intense than that given by glucose and other hexoses and has a secondary absorption maximum in the violet region, not shown by the hexose colour; this makes possible the detection and rough estimation of pentoses.

MATERIALS AND METHODS

Carbazole. The product from L. Light and Co. gave no colour with H_2SO_4 at 100°. Solutions (0·2 and 0·3%, w/v) of carbazole were made up in A.R. ethanol (water content less than 2%). Both were renewed within 3 months of preparation.

Standards. The stock solution of glucuronolactone (L. Light and Co.) was 1 mg./ml. and the dilute solution 0·1 mg./ml. in water. Both were stored at 4°. The dilute standard was renewed within 1 month, and the stock within 3 months of preparation. Glucuronolactone was used in preference to glucuronic acid because of the difficulty of isolating the acid in the free state. Conversion of the lactone into the barium salt or into the free acid did not affect significantly the readings obtained in the standard procedure.

Other carbohydrates. Glucose (May and Baker Ltd.), galacturonic acid, glucosamine hydrochloride, galactono-γ-lactone and gluconic-δ-lactone (L. Light and Co.), ascorbic acid, furfural and xylose (British Drug Houses Ltd.), arabinose, lactose, galactose and fructose (Hopkin and Williams Ltd.) were used in the same concentrations as glucuronolactone. Bornyl and menthyl glucuronosides were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), potassium hyaluronate and chondroitin sulphate from L. Light and Co., and oxidized cellulose from Parke, Davis Ltd. 5-Hydroxymethylfurfural was prepared from sucrose by the method of Haworth & Jones (1944). Barium glucuronate and glucuronic acid were prepared from glucuronolactone by the method of Goebel & Babers (1933).

Standard procedure

Dilution. Maximum accuracy is only attained when the concentrations of both glucuronolactone and glucose in the test solution are within the range 10–100 μg./ml. and the combined amount of the two compounds is not more than 170 μg./ml. For estimations on urine (guinea-pig or human) suitable concentrations within this range are usually obtainable by diluting 1 ml. of urine to 20 ml.

Method. For each test, pairs of 1 ml. samples of appropriately diluted test solutions, of dilute carbohydrate standards and of water blanks are set up in 25 ml. Pyrex tubes, which are placed in water at 4° for 5 min. Then 6 ml. of ice-cold H_2SO_4 (A.R., Hopkin and Williams Ltd.) are added rapidly to each tube and mixed. All tubes are heated in boiling water for 20 min. and then transferred to water at room temperature. After 2 min., 0·2 ml. of 0·2% carbazole is mixed with one of the solutions in each pair. All tubes are kept in an oven at 26° for 2·5 hr. Light absorption is then measured in a Hilger Spekker photoelectric colorimeter

(H. 760) with an Ilford 604 spectrum green filter. Each tube with the carbazole reagent is read against the corresponding blank without the reagent.

A volume (6 ml.) of each solution was then added to 1 ml. of water (this quantity is critical) previously placed in another 25 ml. tube and 0·2 ml. of 0·3% carbazole is mixed with the solutions to which 0·2% carbazole was originally added. All tubes are kept at 26° until 24 hr. after the first addition of carbazole reagent. Light absorption is then measured as before, except that readings are taken with Ilford 605 spectrum yellow–green and with Ilford 601 spectrum violet filters.

The intensity of the colour with glucuronolactone reaches a maximum between 2 and 2·5 hr. after addition of the reagent and thereafter slowly decreases. The colour with glucose slowly increases beyond 24 hr., but after this time the increase is largely offset by an increase in the reagent blank reading.

Standard nomogram. Estimations made on standard mixtures of glucuronolactone, glucose and xylose showed that the absorption readings after 2·5 and 24 hr. corresponded with the sum of the readings to be expected from the amounts of the components of the mixture taken. On this basis the nomogram shown in Fig. 1 was constructed empirically (e.g. Brodetsky, 1938). The nomogram was used as long as the readings given by the two standard solutions (estimated together with the test solution) differed from those shown on the nomogram by less than 3%. Standard readings outside the ±3% limit occurred after the glucose, glucuronolactone or carbazole solutions had been kept for some time. Slight alterations in the standard and reagent blank readings were sometimes found in changing from one sample of sulphuric acid or ethanol to another. If the standard readings cannot be brought to values within 3% of the readings shown on the nomogram by renewing the reagents, then it is necessary to prepare a fresh nomogram.

Mixtures containing pentose. From the absorption reading with the violet filter, in addition to the two readings used for the nomogram, a check can be made on the presence or absence of pentose.

The ratio $R = E_V/(0{\cdot}465\ E_G + E_{YG})$ for both glucose and glucuronolactone was 0·201, where E_V, E_G and E_{YG} were the absorption readings (minus the corresponding reagent blank readings) obtained with the violet (24 hr.), green (2·5 hr.) and yellow–green (24 hr.) filters respectively. The corresponding ratio for xylose was 0·781 and for arabinose 0·713. A ratio greater than 0·201 was thus taken to indicate (in the absence of galacturonic acid, for which $R = 0{\cdot}258$) that pentose was probably present in the material being investigated. If xylose is present it can be calculated, if it is assumed that Beer's law applies, that the concentration of xylose is $E_V(R - 0{\cdot}201)/0{\cdot}58\ wR$, where w is a constant which is calculated by substituting in the expression the figures for a standard solution of xylose. The corresponding expression for arabinose is $E_V(R - 0{\cdot}201)/0{\cdot}512\ wR$. The numerical factors in all these expressions were altered by changes in the quality of the carbazole, ethanol and sulphuric acid.

When the concentration of pentose has been calculated by one of these expressions, the absorption values corresponding with this content of the pentose may be subtracted from E_G and E_{YG}, and the corrected values used to obtain glucose and glucuronolactone concentrations from the standard nomogram.

For glucose and galacturonic acid $R_1 = 0{\cdot}197 = E_V/(0{\cdot}784\ E_G + E_{YG})$; expressions for calculating pentose concentration may be derived if galacturonic is the only uronic acid.

RESULTS

Some results with various carbohydrates are shown in Tables 1 and 2.

Absorption spectra of the coloured complexes. Absorption spectra of the coloured complexes obtained in the standard procedure with a number of compounds were measured with the Hilger Uvispek (H. 700) spectrophotometer. The curves for hydroxymethylfurfural and furfural (after 24 hr.) are shown in Fig. 2. The curves from glucose, galactose and fructose (after 24 hr.) had the same shape and $\lambda_{max.}$ at 545 mμ as that from hydroxymethylfur-

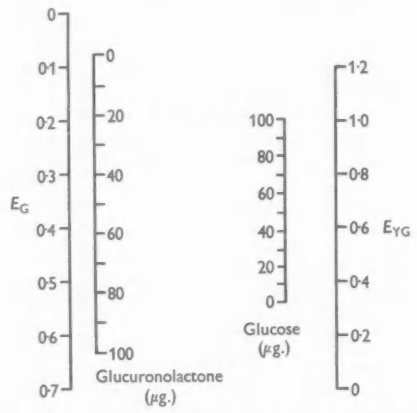


Fig. 1. Nomogram for the estimation of glucuronolactone and glucose from two readings taken with the Spekker absorptiometer; E_G after 2·5 hr., and E_{YG} after 24 hr. by the standard procedure.

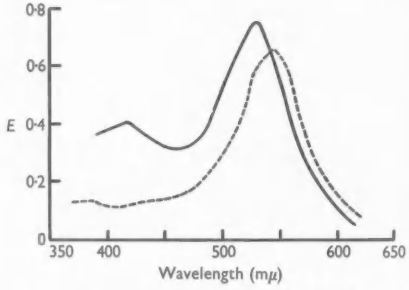


Fig. 2. Absorption spectra of carbazole complexes with hydroxymethylfurfural and furfural treated by the standard procedure. Broken line, 45 μg. of hydroxymethylfurfural; unbroken line, 50 μg. of furfural.

Table 1. *Absorptiometer readings for the coloured complexes obtained by the standard procedure with various compounds*

Substance	Quantity (μmoles)	E at 2·5 hr. Filter 604 (E_G)	E at 24 hr. Filter 605 (E_{YG})	E at 24 hr. Filter 601 (E_Y)
Carbazole blank	—	0·007	0·070	0·034
Glucuronic acid	0·568	0·644	0·372	0·152
Galacturonic acid	0·568	0·537	0·375	0·176
Glucuronolactone	0·568	0·650	0·370	0·154
Barium glucuronate	0·568	0·664	0·375	0·157
Bornyl glucuronoside	0·568	0·549	0·320	0·150
Menthol glucuronoside	0·568	0·581	0·312	0·146
Glucose	0·556	0·060	0·940	0·214
Fructose	0·556	0·060	0·960	0·218
Galactose	0·556	0·051	0·640	0·146
Arabinose	0·556	0·020	0·315	0·213
Xylose	0·556	0·024	0·462	0·346
Glucosamine	0·556	0·030	0·096	0·052
Galactono-γ-lactone	0·556	0·009	0·088	0·044
Gluconic-δ-lactone	0·556	0·012	0·087	0·051
Ascorbic acid	0·556	0·012	0·083	0·046
Lactose	0·292	0·050	0·790	0·198
'Soluble' starch	100 μg.	0·051	1·008	0·228

Table 2. *Results obtained by the standard procedure with solutions containing glucuronolactone, glucose and xylose*

In each experiment eight equivalent samples of 1 ml. each were taken for estimation.

Expt. no.	Amount taken (μg.) Glucurono-lactone	Glucose	Xylose	Amount found (mean ± S.D.) (μg.) Glucuronolactone	Glucose	Xylose
1	100	—	—	99·72 ± 1·65	—	—
2	90	10	—	89·44 ± 1·29	10·41 ± 0·91	—
3	50	50	—	50·11 ± 1·28	49·42 ± 1·35	—
4	10	90	—	10·43 ± 0·45	90·67 ± 1·14	—
5	—	100	—	—	99·87 ± 1·46	—
6	50	50	10	49·45 ± 1·03	48·72 ± 0·99	12·67 ± 1·69
7	50	50	50	49·71 ± 0·99	48·93 ± 1·64	48·99 ± 1·29
8	10	10	100	10·37 ± 0·61	11·92 ± 3·04	98·77 ± 2·02

fural. There was a similar correspondence between the curves for xylose and furfural ($\lambda_{max.}$ 530 and 415 mμ). The curve for glucuronolactone after 2·5 hr. had $\lambda_{max.}$ 525 mμ and after 24 hr. 575 mμ.

Determinations on polysaccharides. The percentages (Table 3) of glucuronic acid found in purified hyaluronate, chondroitinsulphate and heparin, and the molar ratio of sulphate:uronic acid in heparin are within the ranges given by previous workers. [For hyaluronic acid, see Roseman, Moses, Ludowieg & Dorfman (1953) and Kent & Whitehouse (1955); for chondroitinsulphate, see Einbinder & Schubert (1950) and Meyer, Linker, Davidson & Weissmann (1953); for heparin, see Foster & Huggard (1955).] The theoretical percentages calculated from the postulated structures of potassium hyaluronate and calcium chondroitinsulphate are 46·1 and 36·6 respectively. The apparent finding of a small amount of glucose in the heparin preparation was not altered by an attempted purification of the preparation by precipitating with cetylpyridinium chloride (see Scott, 1955).

An estimation (not shown in Table 3) on a soluble fraction of gum tragacanth (prepared by the method of Norman, 1931) showed 51·2% by wt. of uronic acid (as galacturonic) and 43·5% of pentose (as arabinose). The whole gum, on the other hand, showed only 34·8% of galacturonic acid, about 18·2% of pentose (arabinose) and 11·8% of hexose (glucose). These results are in accord with the mixed nature of the gum (see Jones & Smith, 1949).

Determinations on human urine. Urine samples 1, 2, 3, 5 and 6 (Table 4) were all obtained from apparently healthy male Chinese, samples 7 and 8 from female Chinese and samples 4 and 10 from European subjects. Sample 9 was obtained from a male Chinese, aged 9 years, with subacute nephritis,

and contained 0·85 g. of protein/100 ml. Samples 2 and 3 were obtained during urine-concentration tests (e.g. Fishberg, 1939) and thus should presumably have contained dissolved material in a concentration approaching the maximum possible for normal subjects. Urine 1 was obtained after a large intake of fluid by the subject concerned. Urines 6, 7, 8 and 9 were 24 hr. specimens with total volumes of 750, 1075, 620 and 460 ml. respectively.

If a creatinine excretion of 1·7 g./day for each subject is assumed for samples 1–5, the calculated or estimated daily excretion of glucuronic acid for all the samples ranges from 0·065 to 0·67 g./day. This is just within the normal range for European subjects (Brox, 1953; Fishman *et al.* 1951; Deichmann & Thomas, 1943).

The recovery experiments for urine samples 5 and 7 illustrate the effect of insufficient dilution of the

Table 3. *Glucuronic acid and glucose contents of some acidic polysaccharides as estimated by the carbazole reaction*

All the polysaccharide preparations except heparin were dried *in vacuo* over P_2O_5 at 80° for 2 hr. before weighing. Solutions of all the preparations were in water except oxidized cellulose, which was in 0·005 N-NaOH. Sulphate analyses on the solutions of chondroitinsulphate and heparin were carried out in triplicate by the method of Bray, Humphris, Thorpe, White & Wood (1952). Glucose was assumed to be nil if the estimated glucose content of the polysaccharides was not more than 2% by wt. of the estimated glucuronic acid content. Estimated pentose contents of the polysaccharides were in all cases less than 4% of the combined estimated glucuronic acid and glucose contents and were assumed to be nil in calculating the figures given for glucuronic acid and glucose.

Material	Quantity	Glucuronic acid (μmoles)	Glucuronic acid (%)	Glucose (μmoles)
Potassium hyaluronate	360 μg.	0·392	21·1	0·172
Purified hyaluronate*	155 μg.	0·289	36·3	Nil
Chondroitinsulphate (containing 2·95% N)	310 μg.†	0·529	33·1	Nil
Heparin ('Pularin' Evans; 5000 i.u./ml.)	25 i.u.‡	0·403	21·7	0·077
Oxidized cellulose	100 μg.	0·313	60·8	0·122
Bromine-oxidized starch§	140 μg.	0·113	15·6	0·522

* Aqueous potassium hyaluronate (20 mg. in 10 ml.) was shaken with 5 ml. of amyl alcohol–chloroform (1:4, v/v) for 0·5 hr. The mixture was then centrifuged at 2000 rev./min. for 0·5 hr.; about 6 ml. of the clear aqueous layer was mixed with 95% ethanol (30 ml.); this mixture was kept for 6 hr. at 4°, and the resulting precipitate of hyaluronate was separated by centrifuging.

† Contained 0·537 μmole of sulphate.

‡ 0·36 mg. dry wt. Contained 0·941 μmole of sulphate.

§ Prepared by the method of Farley & Hixon (1942).

Table 4. *Concentrations and recoveries of uronic acid and hexose in various urines, as estimated by the carbazole procedure*

Values in columns 5–8 are means and standard deviations obtained from six individual estimations of 1 ml. of diluted urine samples.

Urine	Sp.gr.	Creatinine (mg./ml.)	Dilution of urine for the carbazole reaction (ml./20 ml.)	Estimated concn. in undiluted urine (μg./ml.): Uronic acid (as glucuronolactone)	Estimated concn. in undiluted urine (μg./ml.): Hexose (as glucose)	Recoveries (μg./ml. of diluted urine) after addition of 20 μg. (50 μg. for urine 5): Glucuronolactone	Recoveries (μg./ml. of diluted urine) after addition of 20 μg. (50 μg. for urine 5): Glucose
1	1·005	0·30	2·0	62±5·6	224±10·1	18·9±0·61	19·8±0·89
2	1·033	3·40	1·0	483±14·1	607±32·7	18·5±0·96	19·2±1·29
3	1·029	4·60	1·0	315±11·3	707±28·1	18·2±0·94	20·3±1·42
4	1·010	1·34	1·0	427±9·4	433±29·4	18·5±0·35	19·4±1·27
5	1·024	2·24	0·8	755±23·8	1313±16·3	46·7±1·13	44·0±0·97
5	1·024	2·24	0·4	794±21·2	1306±31·6	48·1±1·07	49·5±0·97
6	1·016	1·85	1·0	348±18·1	561±30·3	18·5±0·31	19·1±1·28
7	1·011	0·78	1·0	55±4·1	1883±72·8	19·6±0·60	15·5±2·17
8	1·018	1·60	1·0	237±8·4	247±7·6	19·2±0·42	20·0±0·92
9	1·013	0·34	1·0	40±3·4	279±17·2	19·1±0·50	18·7±2·05
10	1·020	1·69	1·0	465±19·7	1243±63·2	17·9±1·18	20·1±2·45
10 plus acetoacetate (2 mg./ml.) and acetone (1 mg./ml.)	—	—	1·0	—	—	17·1±0·51	19·6±1·33
10 plus albumin (2 mg./ml.)	—	—	1·0	—	—	18·1±0·85	19·0±1·31
10 plus urea (50 mg./ml.)	—	—	1·0	—	—	18·3±1·31	19·6±1·92

urine on the results obtained. When 0·8 ml. of urine 5 was diluted to 20 ml. the concentration of glucuronolactone plus glucose was above the limit of 170 μg./ml. (see Methods); with urine 7 the final concentration of glucose was higher than 100 μg./ml.

The estimated pentose contents of the urine samples had little statistical significance and are not included in Table 4. In no case was the probability, that the mean of the estimates for a urine sample differed from zero by chance alone, less than 0·09.

One experiment with 40 μg. of added arabinose gave a mean recovery of $32{\cdot}85 \pm 2{\cdot}34$ μg. The estimated concentrations of glucuronolactone and glucose in this urine were 528 ± 19 and 1335 ± 42 μg./ml. respectively. The concentrations found for the urine without added arabinose were 540 ± 24 and 1300 ± 27 μg./ml.

The estimation of pentose by the standard procedure was unreliable in many urines because of variable contributions to the E_V reading by materials other than pentose. Acetone and acetoacetate, for instance, in a concentration greater than 2–3 mg./ml., gave a slight yellowish colour after 24 hr. which absorbed light in the violet region.

Guinea-pig urine. Samples of 24 hr. urine collected over 4 days from nine normal guinea pigs on a dry diet (crushed oats, bran and milk powder with appropriate vitamin and mineral supplements) showed a mean total (free plus combined) glucuronic acid excretion of $3{\cdot}34 \pm$ S.D. 1·67 mg./day/guinea pig and a glucose excretion of $7{\cdot}50 \pm 4{\cdot}70$ mg. The same guinea pigs were each injected subcutaneously with 50 mg. of borneol in olive oil on two separate days. The glucuronic acid and glucose excretions in the 24 hr. after injection were $19{\cdot}24 \pm 6{\cdot}16$ and $7{\cdot}84 \pm 6{\cdot}52$ mg. respectively.

DISCUSSION

The results in Table 2 show that the modified carbazole method can be applied to solutions containing glucuronolactone, glucose and xylose to give estimates of one or all of these constituents over the concentration range for each of 10–100 μg./ml.

Table 1 shows that the differences between the readings obtained with equimolar amounts of glucuronolactone, glucuronic acid and barium glucuronate were not significant in relation to the standard deviations given in Table 2. The readings obtained with bornyl and menthyl glucuronosides were somewhat lower than for an equimolar amount of glucuronolactone, but the magnitudes of any two of the readings were related to the third by almost the same ratios as for glucuronolactone. Table 1 also shows that fructose and soluble starch gave readings which did not differ significantly from those of an equivalent amount of glucose. Fructose gave a pink blank not given by glucose in equimolar concentration, but this colour apparently did not interfere with the development of the colour with carbazole, or the subsequent measurement against the blank. Galactose and lactose gave lower readings than an equivalent amount of glucose, but the magnitudes of any two of the readings were related to the third by almost the same ratios as for glucose. It is thus possible to use the nomogram (Fig. 1) for the estimation of glucuronic acid and its barium salt (and presumably other salts), and of fructose and the glucose in soluble starch.

An attempted purification of the heparin preparation (Table 3), unlike that of the hyaluronate preparation, failed to reduce the low estimated glucose content. The anomalous readings obtained with heparin may be characteristic of the structure of the compound rather than a result of the presence of glucose or some other hexose in the preparation. Dische (1955) states that the hexuronic acid of heparin gives anomalous results in a number of colour reactions. Dische (1947) found that the extinction coefficient of the colour obtained with heparin in the reaction with carbazole was 50% greater than that obtained with an equivalent amount of glucuronic acid. The extinction coefficient obtained for heparin in the present work does not appear to be greater than that which might be expected from the glucuronic acid content calculated from previous analyses on heparin preparations (see Foster & Huggard, 1955).

The figures obtained for gum tragacanth, together with those in Table 2, indicate the possibility that the method may be useful in testing preparations of the plant hemicelluloses, which contain glucuronic or galacturonic acid, glucose or galactose and xylose or arabinose.

The highest concentration of glucose found in nine normal urines was 0·19%. In the present method, non-carbohydrate materials and reducing glucuronosides do not contribute to the figures for glucose. The figures would, however, include, if they were present, other free or combined hexoses which occasionally occur in urine. The recovery of added glucuronolactone and glucose appeared to vary little despite the widely differing specific gravities of the urines. Albumin, urea, or acetoacetate plus acetone, when added in amounts corresponding with the average excess above normal found in pathological conditions, had little effect on the recovery from urine of either glucuronolactone or glucose. The urine of a nephritic subject also showed no decrease in the recoveries. Further recovery experiments, not shown in Table 4, indicated that addition of larger quantities of the ketone bodies decreased the recovery of glucuronolactone and that addition of larger quantities of albumin decreased the recovery of glucose, whereas the addition of larger quantities of urea gave recoveries

of glucose greater than 100%. Dische (1947) found that serum proteins in a concentration of 0·1% produced a 20% decrease in the intensity of the colour given by glucuronolactone. The failure to observe any decrease in the readings obtained with glucuronolactone in the present work may have been due to the use of a test blank, and of 0·2% (instead of 0·1%) ethanolic carbazole.

The similarity of the absorption spectra of the colour complexes of glucose and of hydroxymethylfurfural suggests that the colour from glucose is produced by reaction of carbazole with hydroxymethylfurfural formed from glucose by the action of sulphuric acid. Similarly, the colour from xylose is probably the result of a reaction between furfural and carbazole. Since the absorption spectra of the complexes from glucuronolactone at 2·5 and 24 hr. are both different from the above, and since neither furfural nor hydroxymethylfurfural gives as intense a colour at 2·5 hr. as glucuronolactone, it seems that neither of these compounds is principally responsible for the colour obtained with glucuronolactone and carbazole.

SUMMARY

1. A modification of the carbazole method for uronic acids has been adapted for the estimation of glucuronolactone, glucose and xylose in solutions containing any or all of these compounds in the concentration range 10–100 μg./ml. The results were reproducible to within ± 4 μg. except for some mixtures containing xylose.

2. The method gave good recoveries of glucuronolactone and glucose added to urine. These recoveries were not affected by the addition of albumin, ketone bodies, urea or arabinose in limited quantities.

3. The method gave figures for the concentrations of glucuronic acid in chondroitinsulphate, hyaluronate and heparin preparations, and in urine, which were of the same order as previously published figures obtained by other methods.

4. Hydroxymethylfurfural is probably responsible for the colour given by glucose, and furfural for that given by xylose. The material responsible for the colour given by glucuronic acid does not appear to be either of these compounds.

I am indebted to Mr G. M. Ching for the creatinine determinations.

REFERENCES

Bray, H. G., Humphris, B. G., Thorpe, W. V., White, K. & Wood, P. B. (1952). *Biochem. J.* **52**, 412.
Brodetsky, S. (1938). *A First Course in Nomography.* London: Bell and Sons.
Brox, G. (1953). *Dtsch. Z. Verdau.-u. Stoffwechselkr.* **13**, 99.
Deichmann, W. B. & Thomas, G. (1943). *J. industr. Hyg.* **25**, 286.
Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Dische, Z. (1955). *Methods of Biochemical Analysis*, vol. 2. New York: Interscience.
Einbinder, J. & Schubert, M. (1950). *J. biol. Chem.* **185**, 725.
Farley, F. F. & Hixon, R. M. (1942). *Industr. Engng Chem. (Industr.)*, **24**, 677.
Fishberg, A. M. (1939). *Nephritis and Hypertension.* London: Baillière, Tindall and Cox.
Fishman, W. H., Smith, M., Thompson, D. B., Bonner, C. D., Kasdon, S. C. & Homburger, F. (1951). *J. clin Invest.* **30**, 655.
Foster, A. B. & Huggard, A. J. (1955). *Advanc. Carbohyd. Chem.* **10**, 335.
Goebel, W. F. & Babers, F. H. (1933). *J. biol. Chem.* **100**, 573.
Haworth, N. & Jones, W. G. M. (1944). *J. chem. Soc.* p. 667.
Jones, J. K. N. & Smith, F. (1949). *Advanc. Carbohyd. Chem.* **4**, 243.
Kent, P. W. & Whitehouse, M. W. (1955). *Biochemistry of the Amino sugars.* London: Butterworths.
Meyer, K., Linker, A., Davidson, E. A. & Weissmann, B. (1953). *J. biol. Chem.* **205**, 611.
Norman, A. G. (1931). *Biochem. J.* **25**, 200.
Roseman, S., Moses, F. E., Ludowieg, J. & Dorfman, A. (1953). *J. biol. Chem.* **203**, 213.
Scott, J. E. (1955). *Biochim. biophys. Acta*, **18**, 428.

Latent Phenolase in Extracts of Broad-Bean (*Vicia faba* L.) Leaves

1. ACTIVATION BY ACID AND ALKALI

By R. H. KENTEN
Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts

(*Received* 8 *March* 1957)

Latent phenolase activity has been found in extracts of eggs of the grasshopper *Melanoplus differentialis* (Bodine & Allen, 1938*a*, *b*), mealworm *Tenebrio molitor* (Bodine & Allen, 1941), *Drosophila* spp. (Ohnishi, 1954; Horowitz & Fling, 1955) and crayfish serum (Bodine & Allen, 1941). The extensive investigations of Bodine and co-workers (Bodine & Boell, 1935; Bodine, Allen & Boell, 1937; Allen, Ray & Bodine, 1938; Allen & Bodine, 1941; Allen, Otis & Bodine, 1942; Bodine & Tahmisian, 1943; Bodine, Tahmisian & Hill, 1944) with extracts and preparations from grasshopper eggs have shown that active phenolase is released by treatment with certain organic solvents (e.g. acetone, chloroform),

certain detergents, by heating to 60–70°, or by brief exposure to weak alkali (about pOH 4). They have obtained evidence that these effects are due to the presence in the extracts of egg of a copper–protein which is converted into an active phenolase by the various treatments.

Kenten (1955) has reported that much of the phenolase of extracts of broad-bean leaves is present in a masked form and that release of active enzyme follows treatment with certain proteolytic enzymes or anionic wetting agents. The nature of the masked phenolase is not yet clear, but the present work describes some treatments which bring about activation of the phenolase.

MATERIALS AND METHODS

Plant material. Most of the broad-bean leaves used were taken from plants grown in a heated greenhouse in John Innes Compost, but occasionally leaves from garden plants were used. Other plant material was found growing wild or in gardens.

Plant extracts. The plant material was ground with sand and an equal weight of water previously cooled to 0° in a chilled mortar. The slurry was squeezed by hand through strong cotton cloth and the water extract collected in a cylinder surrounded by ice and water.

Precipitation and centrifuging. Unless otherwise stated, precipitations were made at 0–4° with $(NH_4)_2SO_4$. Ethanol and acetone were cooled to −10° and the temperature was maintained below 4° during the precipitation. Centrifuging was carried out in an MSE angle-head centrifuge (Measuring and Scientific Equipment Ltd.) at 0–4°.

Phenolase activity. This was followed manometrically by measuring the rate of O_2 uptake at 25° when the preparations acted on DL-β-3:4-dihydroxyphenylalanine (DOPA), catechol, adrenaline or *p*-cresol. The volume of the reaction mixtures was 3 ml., the gas phase was air and KOH was present in the centre cup. Phenolase activity was also estimated by a method essentially similar to that of Horowitz & Shen (1952). The rate of formation of the red initial oxidation product of DOPA was followed with an EEL colorimeter (Evans Electroselenium Ltd.) with a Bright Spectrum blue–green filter no. 623, maximum transmission at 495 mμ. Reaction mixtures consisted of 3 mg. of DOPA and enzyme in a total volume of 6 ml., 0·025 M with respect to both phosphate and citrate at pH 6. Under these conditions there was no gross departure from a linear rate of oxidation over the first 3·5 min. of the reaction and the rate of oxidation was proportional to the amount of enzyme used, provided that the change on the EEL scale was less than 30 units. For routine determinations 0·05–0·5 ml. of enzyme was added at zero time to the other reagents and, after thoroughly mixing, readings were taken at 20 and 200 sec., and the increase in light absorption over this period was taken as a measure of the enzyme activity. Blank determinations with enzyme previously heated for 5 min. at 100° always gave very small values, rarely exceeding 1 EEL unit in 200 sec.; where the amount of protein was large, a small increase in transparency was observed owing to the settling out of coagulated protein unless the solution was frequently stirred. On no occasion was a red coloration seen in the blank determinations.

Estimation of β-3:4-dihydroxyphenylalanine. This compound was estimated by the colorimetric procedure of Kendal (1949) with an EEL colorimeter and filter no. 623.

Buffers. Phosphate buffers were prepared from solutions of KH_2PO_4 and KOH, citrate and acetate buffers from solutions of the acids and NaOH.

Ammonium sulphate. For most of the work British Drug Houses Ltd. Laboratory Reagent grade $(NH_4)_2SO_4$ was used.

pH *measurements.* These were made with a Cambridge or Beckman 'General Purpose' glass electrode. The Beckman electrode was used exclusively at pH values above 9 since it has a low Na^+ ion error and a negligible potassium error at the highest pH values measured.

EXPERIMENTAL AND RESULTS

It is well known that when broad-bean leaf sap is left to stand it soon begins to darken, first at the surface and then through the whole fluid, which finally resembles ink. It is believed that the production of melanin by the oxidation of tyrosine or DOPA is responsible for the blackening of the sap. Guggenheim (1913) isolated L-DOPA from broad-bean pods (2·5 g./kg. of fresh pod), and in the present work the DOPA content of a number of different samples of broad-bean leaves was found to vary between 3·5 and 9·4 g./kg. fresh wt. There is therefore ample DOPA present to account for the observed blackening of leaf sap. All the water extracts of broad-bean leaves prepared in the present work turned black on standing, although a considerable variation in the rate at which different batches darkened was observed. During attempts to prepare an oxidase free from DOPA from such water extracts it was often found that repeated precipitation with $(NH_4)_2SO_4$ gave a preparation with much greater activity towards DOPA or catechol at pH 6 than did a single precipitation.

Effect of repeated precipitation with ammonium sulphate. A water extract of broad-bean leaves was centrifuged at 8000 *g* for 20 min. and the pellet discarded. All subsequent steps were carried out at room temperature. A portion of the supernatant was set aside and the rest saturated with $(NH_4)_2SO_4$. The precipitate was centrifuged off and suspended with water to a volume equal to that of the supernatant taken for precipitation. A sample of this suspension [first $(NH_4)_2SO_4$ ppt.] was taken and the rest reprecipitated by saturating with $(NH_4)_2SO_4$. The precipitate from centrifuging was suspended in water to a volume equal to that of the suspension taken for precipitation. After sampling this second $(NH_4)_2SO_4$ ppt. the cycle was repeated until all the fractions had been obtained. The rate of oxidation of added DOPA or catechol by the fractions at pH 6 was then estimated (Table 1).

The results show that a large increase in the phenolase activity is brought about by repeated precipitation with $(NH_4)_2SO_4$. The activity of all of the fractions was virtually completely destroyed by

heating for 5 min. at 100° before testing. The magnitude of the effect brought about by repeated precipitation was extremely variable, often being much less than that shown in Table 1.

When active phenolase was obtained from water extracts by repeated precipitation with $(NH_4)_2SO_4$ it could be precipitated with only slight loss of activity by the addition of 2 vol. of acetone or ethanol. However, repeated precipitation of centrifuged water extracts with 2 vol. of acetone or ethanol failed to bring about an increase in phenolase activity at pH 6. The pH of the water extracts was in the neighbourhood of pH 6, and when repeated precipitation with $(NH_4)_2SO_4$ was carried out in the presence of phosphate buffer at pH 6 the increases in activity obtained were negligible compared with those found with extracts without added buffer. With unbuffered solutions, as each cycle of precipitation is carried out the pH falls owing to the acidity of the $(NH_4)_2SO_4$. The results of the following experiments show that exposure of the bean-leaf extracts to suitable acidities does in fact increase the phenolase activity at pH 6 and that exposure to alkaline conditions has a similar effect.

Effect of acid and alkali. For these experiments a preparation free from the naturally occurring polyphenols was made from a centrifuged water extract of broad-bean leaf by dialysis for 24 hr. at 0–4° against large volumes of buffer, pH 6, 0·004 M with respect to phosphate and citrate. Portions of the dialysed extract (40–50 ml.) were held below 10° and N-H_2SO_4 or N-KOH was added dropwise with brisk stirring. Samples (2 ml.) were withdrawn at various pH values into cold (0–5°) 0·2 M-phosphate–citrate (1 ml.), of pH 6 when the samples were alkaline and pH 6·5 when the samples were acid. The pH of these mixtures was 6–6·5 except when the pH of the dialysed extract was outside the range 2–12, when further adjustment with acid or alkali was necessary. The whole operation was carried out rapidly and the addition of acid or alkali and sampling was complete within 10–15 min. Within 1 hr. of preparation the phenolase activity of the samples was estimated colorimetrically at room temperature (18·5–20°). The results of one such experiment are given in Fig. 1. The values of phenolase activity are the increase in light absorption, in EEL-scale units, which would be brought about by the action of 1 ml. of the samples of dialysed water extract (corrected for dilution by the addition of KOH or H_2SO_4 and the phosphate–citrate buffer) under the standard conditions. All the values given in Fig. 1 were obtained with a single extract. Not all of the samples were tested for thermolability but the most active samples (those exposed to a pH or pOH of 2–4) were completely inactivated by heating for 2–3 min. at pH 6–6·5. Furthermore, if the dialysed extract was heated before exposing it to acid and alkaline pH no phenolase activity was found. The results (Fig. 1) show that within limits, exposure of the water extracts to acidity or alkalinity outside the range pH 6–7 increases the phenolase activity towards DOPA at pH 6. With different batches of extract the results varied somewhat in the magnitude of the effects and the ratio of the maximum activities reached at acid and alkaline pH values. In general, the maximum activity released by exposure to acid pH was greater than that obtained by exposure to alkaline pH. The maximum activities were obtained on the acid side in the range pH 2·8–3·8 and on the alkaline side pOH 2·2–3·2; with increase in acidity or alkalinity outside this range the activity decreased, suggesting that it was destroyed by too

Table 1. *Effect of repeated precipitation with ammonium sulphate on the phenolase activity of water extract of broad-bean leaves*

Reaction mixtures consisted of 3 mg. of DOPA or 5 mg. of catechol in 0·1 M-phosphate at pH 6 in the main vessel. After equilibration 0·5 ml. of water extract or $(NH_4)_2SO_4$ precipitate was added from the side arm. Water extracts contained about 3 g. of DOPA/l. and had O_2 uptakes in the absence of added phenol of 7–8 μl. of O_2/0·5 ml. in the first 30 min. Uptakes with the $(NH_4)_2SO_4$ precipitates in the absence of added phenol varied between 4·5 and 6·5 μl. of O_2 in 30 min. Blank determinations with heated extract or precipitate were less than 4 μl. of O_2 in 30 min.

	Increased O_2 uptake (μl./0·5 ml. in the first 30 min.)	
Fraction	Expt. 1. DOPA as substrate	Expt. 2. Catechol as substrate
Centrifuged water extract	2	4
1st $(NH_4)_2SO_4$ ppt.	5·5	20·5
2nd $(NH_4)_2SO_4$ ppt.	18·5	36
3rd $(NH_4)_2SO_4$ ppt.	91·5	56·5
4th $(NH_4)_2SO_4$ ppt.	226	—
5th $(NH_4)_2SO_4$ ppt.	176·5	—

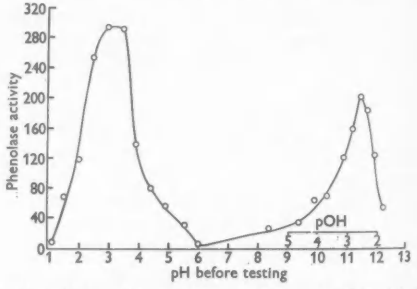


Fig. 1. Effect on the phenolase activity at pH 6 of brief exposure of dialysed water extracts of broad-bean leaf to different pH values at 5–10°.

extreme conditions. In alkaline solutions the pH decreases as the temperature increases, owing to the variation of pK_w with temperature, whereas the pOH, which is the important factor, remains unaltered. It is for this reason that the values given in Fig. 1 for pH have been calculated to the pH at 25° with the values for K_w given by Harned & Owen (1939). At 25°, $pK_w = 14$ and the $pOH = 14 - pH$. The effect of temperature on the pH of the acidified dialysed water extracts was small and differences between 5° and 25° were less than 0·2.

Effect of incubating dialysed water extracts of broad-bean leaves in the absence and presence of ammonium sulphate on the phenolase activity

It was found that when the precipitates, obtained from water extracts of broad-bean leaves, by saturation with $(NH_4)_2SO_4$, were suspended in water and allowed to stand at room temperature their phenolase activity at pH 6 often increased. The rate of increase in activity was found to be dependent on temperature, being more rapid at 37° than at 0°. Little or no increase was observed when the suspensions were stored frozen at −18° for 14 days, but subsequent incubation at 37° brought about a large increase in activity. A typical set of results obtained with a second $(NH_4)_2SO_4$ ppt. is shown in Table 2. No increase in activity was obtained when the preparation was heated for 5 min. at 100° before incubating for 15 hr. at 37°. Heating the fractions stored at the various temperatures for 5 min. at 100° before testing with DOPA destroyed 95–100 % of the activity.

Experiments with dialysed water extracts of broad-bean leaves showed that the increase in phenolase activity obtained on incubation at 37° depended on the pH and the presence of $(NH_4)_2SO_4$ (Table 3).

Mixtures were made containing 6 ml. of dialysed water extract with and without the addition of 0·75 g. of $(NH_4)_2SO_4$ in a total volume of 10 ml., 0·03 M with respect to both phosphate and citrate at pH 3, 4, 5 or 6. A few drops of toluene were added to inhibit the growth of micro-organisms. (No evidence that either toluene or chloroform has any activating effect was obtained.) Samples were taken as soon as the mixtures were prepared and the activity towards DOPA at pH 6 was measured manometrically. It was found that adjustment of the pH before measuring the activity was not necessary, as the pH of the manometric reaction mixtures was not affected by the addition of the 0·1 ml. samples tested. The dialysed-extract mixtures were then incubated at 37° and samples were taken at different times, and the activity towards DOPA at pH 6 was measured. The results show that initially the highest activity is present in those samples at pH 4. On incubation, however, the largest activity is reached in the sample containing $(NH_4)_2SO_4$ at pH 5. In the absence of $(NH_4)_2SO_4$ an increase in activity is observed only with the mixture at pH 5 and the effect is small compared with that obtained in the presence of $(NH_4)_2SO_4$ at the same pH. At pH 3 the activity is markedly unstable and in other experiments where phenolase was estimated colorimetrically with DOPA it was found that 0·5–1 hr. at 37° and pH 3 destroyed 95–100 % of the activity. Prolonged incubation at any of the experimental pH values leads finally to complete loss of phenolase activity regardless of the presence of $(NH_4)_2SO_4$. If the samples incubated at pH 6 for 72 hr. are then acidified to pH 4 little or

Table 2. *Effect of ageing at different temperatures on the activity of an ammonium sulphate precipitate towards DOPA at* pH 6

A second $(NH_4)_2SO_4$ ppt. from a water extract of broad-bean leaves was stored at different temperatures and the activity towards DOPA at pH 6 was measured manometrically under the conditions of Table 1.

Conditions of storage	Increased O_2 uptake in first 30 min. (μl./0·4 ml. of fraction)
4 hr. at 0°	12
15 hr. at 0°	54
15 hr. at 37°	235
15 hr. at 37°, then 7 days at 0°	443
8 days at 0°	237
8 days at −18°	13

Table 3. *Phenolase activity at* pH 6 *of dialysed water extracts of broad-bean leaves after incubation at acid* pH *in the absence and in the presence of ammonium sulphate*

A dialysed water extract of broad-bean leaves was incubated at 37° under the conditions described in the text, and the activity towards DOPA was measured at the stated times. Manometric reaction mixtures consisted of 3 mg. of DL-DOPA in buffer pH 6, 0·1 M with respect to phosphate and citrate. At zero time 0·1 ml. samples of the dialysed extract mixtures were added from the side-arm. The values given are corrected for the blank O_2 uptakes when DOPA was omitted or when the samples were heated (5 min. at 100°) before testing. These uptakes were less than 4 μl. of O_2 in 10 min.

pH of incubation ...	3		4		5		6	
$(NH_4)_2SO_4$	−	+	−	+	−	+	−	+
Time of incubation (hr.)	Uptake of O_2 (μl. in 10 min.)							
0	17	12	59	53	9	21	5	1
6	1	1	49	63	15	67	2	4
24	−1	−1	11	24	15	79	5	12
48	0	2	0	−2	14	62	−1	6
72	0	2	0	2	−1	10	2	1

no increase in activity is observed, which suggests that the material from which activity is derived has been altered by the incubation.

Effect of ammonium sulphate on the stability of the phenolase activity released by acidifying dialysed water extract of broad-bean leaves

The phenolase activity released by brief exposure of dialysed water extracts of broad-bean leaves to acid pH is unstable in phosphate–citrate buffer at pH 6·5 even at 0–5°. However, the presence of $(NH_4)_2SO_4$ markedly reduces the rate of loss of the activity (Table 4).

Buffer solutions at pH 6·5, 0·1 M with respect to phosphate and citrate and containing varying amounts of $(NH_4)_2SO_4$, were made up. A sample of dialysed water extract of broad-bean leaves was then acidified to pH 3·3 at 0–5° by the gradual addition of 0·2 M-H_2SO_4, with brisk stirring. Samples (2 ml.) of the acidified extract were immediately added to samples (4 ml.) of the buffer solutions which had been cooled previously to 0–5°. The activity of these mixtures towards DOPA was then determined colorimetrically at pH 6. The activity was again estimated after the mixtures had been stored at 0–5° for 1, 3 and 22·5 hr. The final pH of these mixtures was in the range 6·4–6·5. Control experiments in which samples of the dialysed extract before acidifying were added to the phosphate–citrate buffer mixtures had a barely measurable phenolase activity (about 50 times less than that of the acidified-extract mixtures) and showed no significant change on storing at 0–5° for 22·5 hr.

The results (Table 4) show that at pH 6·5 the activity is more stable in the presence of $(NH_4)_2SO_4$, and that the stability increases with increase in $(NH_4)_2SO_4$ concentration. This effect of $(NH_4)_2SO_4$ explains why incubation of dialysed extracts at pH 5 in the presence, but not in the absence, of $(NH_4)_2SO_4$ brings about a large increase in the phenolase activity. It is probable that at pH 5 two processes, formation of active phenolase and destruction of active phenolase, are taking place. Assuming that $(NH_4)_2SO_4$ has a similar protective action at pH 5 as at pH 6·5, then the presence of $(NH_4)_2SO_4$ would reduce the rate of destruction of the active phenolase and so favour its accumulation. The increase in activity observed when suspensions of $(NH_4)_2SO_4$ precipitates from water extracts of broad-bean leaves are incubated is probably dependent on $(NH_4)_2SO_4$ retained by the precipitates.

Effect of pH *on reaction velocity with various substrates and after various treatments of enzyme*

From centrifuged water extracts of broad-bean leaves, second $(NH_4)_2SO_4$ precipitates were prepared as described previously except that the preparation was held below 5° throughout. When DOPA and adrenaline were used as substrates part of the precipitate was stored frozen at −18° while the rest was incubated in the presence of toluene at 37°. The rate of oxidation of the two substrates at different pH values was then tested manometrically. With *p*-cresol the preparation was tested as soon as it was prepared and again after incubation.

Table 4. *Effect of ammonium sulphate on the stability of the phenolase activity released by acidification of dialysed water extract of broad-bean leaf*

Mixtures containing acidified dialysed water extract of broad-bean leaf, $(NH_4)_2SO_4$ and phosphate–citrate buffer at pH 6·5 were prepared as described in the text and stored at 0–5°. The activity of these mixtures towards DOPA at pH 6 was measured colorimetrically at 18–19·5° at the stated times.

Final concn. of $(NH_4)_2SO_4$ (M) ...	0	0·17	0·5	1·0	2·3
Time of storage (hr.)	Loss of activity (%)				
1	20	23	14	11	0
3	40	36	27	15	0
22·5	70	61	40	19	0

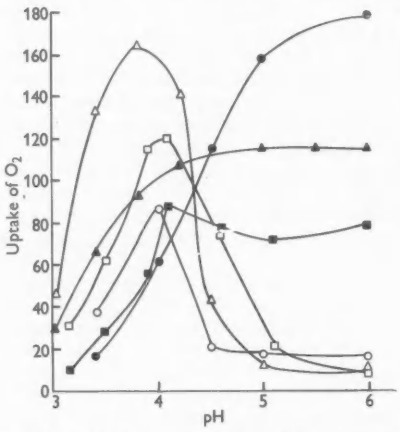


Fig. 2. Effect of pH on the oxidation of phenols by second $(NH_4)_2SO_4$ precipitates from water extracts of broad-bean leaves. DOPA as substrate, enzyme preparation *A*: △, frozen for 45 hr.; ▲, incubated at 37° for 45 hr. Adrenaline as substrate, enzyme preparation *B*: ○, frozen for 30 hr.; ●, incubated at 37° for 40 hr. *p*-Cresol as substrate, enzyme preparation *C*: □, freshly prepared; ■, incubated at 37° for 40 hr. Experiments in phosphate (0·08 M)–citrate (0·08 M) buffer at a phenol concentration of 1 g./l. With DOPA and adrenaline as substrates the O_2 uptake in the first 15 min. with 0·1 ml. and 0·2 ml. of preparation is plotted against the final pH. With *p*-cresol the O_2 uptake in the first hr. with 0·1 ml. of preparation is plotted.

The results (Fig. 2) show that with frozen or freshly prepared precipitates appreciable activity is found only below pH 5, the optimum with all three substrates being in the neighbourhood of pH 4. After incubation the shape of the pH curve changes and the highest activities are found at pH 5–6 with adrenaline and at pH values above 4 with DOPA and *p*-cresol. With *p*-cresol, at all of the pH values tested there was a lag period of about 10–15 min. before the O_2 uptake started. The acid pH optimum found with the frozen or freshly prepared precipitates is probably a consequence of the activation of the latent phenolase brought about by the acid conditions.

The variation of activity with pH for all three substrates was studied under the same experimental conditions with a preparation from potato tuber (*Solanum tuberosum* L.) made in the same way as the broad-bean leaf second $(NH_4)_2SO_4$ precipitate. With this potato phenolase preparation there was little or no activity at pH values below 4 and with all three substrates the rate of oxidation increased with increase in pH values from 4 to 6. There was a 10 min. lag period with *p*-cresol as substrate before the O_2 uptake commenced. Similar experiments with a second $(NH_4)_2SO_4$ precipitate from a water extract of red-clover leaves [*Trifolium pratense* var. *sativum* (Crone) Schreb.] and with a crude chloroplast preparation (Arnon, 1949) from spinach-beet leaves (*Beta vulgaris* L.) with adrenaline as substrate showed that the activity increased with increase in pH value from 3·5 to 6. Phenolase has been reported absent from pea seedlings (Kenten & Mann, 1952) and a second $(NH_4)_2SO_4$ precipitate from pea leaves made in the same way as that from broad-bean leaves showed no activity with adrenaline over the range pH 3–6, either before or after incubation at 37° for 18 hr. in the presence of toluene.

These results suggest that no latent phenolase was present in the potato, clover, spinach-beet and pea preparations.

Tests for the presence of a phenolase inhibitor in the water extracts of broad-bean leaves

The experiments so far have shown that activation of an enzyme occurs; this might occur by the removal of an inhibitor. Accordingly, water extracts were tested together with ultrafiltrate from the extract to find out if an inhibitor of small molecular size was involved, and also with boiled extract, since boiling might liberate an inhibitor from an enzyme–inhibitor complex present in the extract.

Portions of the supernatant prepared from a water extract of broad-bean leaves centrifuged at 8000 *g* for 20 min. were treated in one of the following ways: (*a*) heated for 5 min. at 100°; (*b*) filtered through cellophan at 0° in an apparatus of the type described by Paterson, Pirie & Stableforth (1947), the filtrate passing in the first 4–5 hr. being used; (*c*) precipitated twice by saturation with $(NH_4)_2SO_4$ as described previously, and incubated at 37° in the presence of toluene for 18 hr. to give an active phenolase preparation. Fractions (*a*) and (*b*) and a portion of the original supernatant were stored at 0° until the incubation of the $(NH_4)_2SO_4$ precipitate was complete. The amount of DOPA present in the various fractions was then estimated. No DOPA was detected in the $(NH_4)_2SO_4$ precipitate and the amounts present in the extract, boiled extract and ultrafiltrate were found to be 3·1, 3·1 and 3·0 mg./ml. in the experiment given in Table 5. The rate of oxidation of the DOPA present in these fractions by the incubated $(NH_4)_2SO_4$ precipitate was then followed manometrically by measuring the O_2 uptake and by estimating the disappearance of DOPA colorimetrically. The O_2 uptakes when the incubated $(NH_4)_2SO_4$ precipitate acts on water extract, boiled water extract or ultrafiltrate are about 90 % of that obtained when it acts on added pure DOPA. The disappearance of DOPA from the extract fractions is, however, only about 70 % of that found with pure DOPA. This discrepancy is probably due to the presence of substances (e.g. ascorbic acid) in the extract fractions which are capable of reducing the oxidation product, DOPA quinone, to DOPA. Thus a larger O_2 uptake would be obtained for a given loss of DOPA in these crude systems. The true inhibition of phenolase action by the extract fractions is therefore most likely to

Table 5. *Oxidation of DOPA present in fractions of a water extract of broad-bean leaf by broad-bean-leaf phenolase*

Reaction mixtures comprised: in the main vessel 3 mg. of DOPA or amounts of extract, boiled extract or ultrafiltrate containing 3 mg. of DOPA, in 0·1 M-phosphate at pH 6. After equilibration 0·3 ml. of incubated $(NH_4)_2SO_4$ precipitate [fraction (*c*), see text] was added from the side arm. After 1 hr. an equal volume of 4 % (w/v) metaphosphoric acid was added to the reaction mixtures and the DOPA content estimated. The values for DOPA oxidized were obtained by difference between the DOPA content of the complete reaction mixtures and those from which the $(NH_4)_2SO_4$ precipitate had been omitted. Correction has been made for the blank O_2 uptake of extract (14 μl.), boiled extract (7 μl.), ultrafiltrate (6 μl.), DOPA (2 μl.) and the $(NH_4)_2SO_4$ precipitate (5 μl.).

Fraction used as substrate	DOPA oxidized (mg.)	O_2 uptake (μl./hr.)
DOPA	1·14	203
Centrifuged water extract	0·81	185
Centrifuged water extract, heated at 100°	0·78	180
Ultrafiltrate	0·83	176

be that calculated from the measurements of the O_2 uptake, i.e. about 10%.

Effect of inhibitors. Phenolases from several sources have been shown to be copper-protein enzymes (e.g. Mallette, 1950) and their activity is inhibited by metal-complexing agents such as diethyldithiocarbamate (Dawson & Tarpley, 1951). The effect of a number of such complexing agents has been tested at pH 6 with the second $(NH_4)_2SO_4$ precipitate from a water extract of broad-bean leaves, activated before testing by incubation in the presence of toluene for 18 hr. The results (Table 6) show that agents which are known to inhibit the action of phenolases (Dawson & Tarpley, 1951) inhibited the broad-bean phenolase preparation. This suggests that, like the known phenolases, broad-bean phenolase is a metalloprotein enzyme.

DISCUSSION

The results of the present work show that the phenolase activity at pH 6 of preparations from broad-bean leaves can be greatly increased by brief exposure to mildly acid (pH 3–3·5) or alkaline (pOH 2·5–3) conditions. In the presence, but to a much less extent in the absence, of ammonium sulphate large increases in the phenolase activity at pH 6 are brought about when dialysed extracts of broad-bean leaves are incubated at 37° at about pH 5. These facts provide an explanation for the increase in activity which is observed when water extracts of broad-bean leaves are repeatedly precipitated with ammonium sulphate. The acidity of the ammonium sulphate causes the pH to fall to the neighbourhood of pH 5, which brings about some increase in activity, and in the time which elapses between the preparation of the fractions and testing a further increase in activity takes place.

Studies of the pH–activity relationships before and after treatment show that before activation preparations have an optimum activity in the neighbourhood of pH 4, but little activity at pH 5–6. In general, after activation the activity increases with increase in pH up to pH 6 with no definite optimum pH.

There appear to be two possible explanations of the results. On the one hand it is possible that the pH–activity curves obtained before treatment are those of the native phenolase and that the change in the curve is due to modification of the enzyme protein. In agreement with this possibility all of the treatments which lead to an increase in the phenolase activity at pH 6, namely treatment with acid and alkali, and incubation at pH 5 in the presence of ammonium sulphate, are likely to lead to modification of the properties of a labile protein. On the other hand it is possible that the phenolase enzyme in the extracts is present in a masked form from which it is liberated by the various treatments. It has been shown that brief exposure of the broad-bean-leaf preparations to acid pH increases the phenolase activity at pH 6 and that the activity at pH 6 of the preparation increases with decrease in the pH, to which it has been exposed until about pH 3 is reached. From these data and the pH–activity curves of the activated preparations it would be expected that the untreated preparations would show an acid pH optimum in the neighbourhood of pH 4. The pH–activity curves of the untreated preparations would then be the resultant of the extent of liberation of active enzyme and its activity at the particular pH value. Thus these results suggest that the presence of a masked phenolase in the untreated extracts is the more likely possibility.

Apart from the classical proteolytic zymogens many examples of such masked activity are now known, and a number of them are cited by Swartz, Kaplan & Frech (1956). These authors have demonstrated that in extracts of a number of micro-organisms two enzymes (a diphosphopyridine-nucleotide pyrophosphatase and a 5′-nucleotidase) are present largely in an inhibited state. They have obtained evidence that the enzymes are present in the extracts in combination with a protein inhibitor. Presumably owing to the difference in the stability of the enzyme and inhibiting protein towards heat and acid, active enzyme can be liberated by brief heating at 100° or by acid treatment (pH 1–2 for 10 min.). Swartz *et al.* (1956) suggest that the enzyme and inhibitor are bound by a salt type of linkage.

It has been reported previously (Kenten, 1955) that incubation with certain proteolytic enzymes or treatment with certain anionic wetting agents brings about a large increase in the phenolase activity at pH 6 of water extracts of broad-bean leaves. These results and the results of the present work can be explained by assuming that in the

Table 6. *Effect of inhibitors on broad-bean-leaf polyphenolase*

Manometric reaction mixtures were similar to those described in Table 3. The inhibition was calculated from the uptakes obtained in the first 10–15 min. of the reaction.

Inhibitor	Concn. (mM)	Inhibition (%)
Diethyldithiocarbamate	1·0	93
	0·1	81
Potassium ethyl xanthate	10·0	90
	1·0	49
Salicylaldoxime	1·0	49
Sodium azide	1·0	47

extracts there is either a phenolase–inhibitor complex, in which the components are bound through interaction of ionic groups, or a phenolase precursor (prophenolase). If the former assumption is correct then H^+ ion, OH^- ion and the anionic wetters, by virtue of their capacity to combine with proteins with accompanying alteration of the net charge, would be capable of dissociating such a complex. If the inhibitor is released in this way it must be unstable, as it has not been possible so far to demonstrate that the release of active enzyme is reversible, which suggests that dissociation must be accompanied by modification of the inhibitor. If prophenolase is present in the extracts then the agents which lead to activation could act by modifying the prophenolase molecule in such a way that the active centre is unmasked.

The presence of a phenolase inhibitor in extracts of broad-bean leaves has been sought, but such an inhibitor was not found to be present in amounts sufficient to account for the results described. Attempts to separate an inhibiting protein fraction from the extracts by fractionation with ammonium sulphate have not been successful.

Whatever the state of the masked phenolase in the extracts the question arises whether it is present as such *in vivo* or whether it is formed during the extraction process. It is not clear how the answer to this question can be found experimentally. The possibility of regulation of metabolism by cellular synthesis of specific enzyme inhibitors has been often suggested (e.g. Swartz *et al.* 1956). In support of this suggestion are the several known examples of species-specific enzyme–inhibitor relationships involving protein inhibitors (e.g. Swartz *et al.* 1956). Recognition of this type of phenomenon is comparatively recent, and both it and the conversion of proenzyme into enzyme may prove to be important mechanisms of cellular regulation.

Preliminary tests with extracts from a limited number of different plants have not so far revealed the presence of a masked phenolase with the properties of that found in broad-bean-leaf extracts. This may be owing to the extreme ease with which active phenolase is liberated from the masked form in those species tested. Masked phenolase has been found in extracts from grasshopper eggs, mealworms, extracts of *Drosophila* and crayfish serum, and possibly in extracts of *Neurospora* (Horowitz & Shen, 1952) since these show an increase in phenolase activity on standing. It seems unlikely, therefore, that the broad bean will prove to be unique among the higher plants in giving extracts with latent phenolase activity.

SUMMARY

1. Water extracts of broad-bean leaves contain much latent phenolase activity.

2. Active phenolase is released by brief exposure of the extracts to acid (pH 3–3·5) or alkaline (pOH 2·5–3) conditions, or by incubating in the presence of ammonium sulphate at about pH 5.

3. Evidence has been obtained that the extracts contain little material capable of inhibiting broad-bean phenolase.

4. The nature of the latent phenolase has not been elucidated but it is suggested that either a phenolase precursor or a phenolase–protein inhibitor complex is present in the leaf extracts.

REFERENCES

Allen, T. H. & Bodine, J. H. (1941). *Science*, **94**, 443.
Allen, T. H., Otis, A. B. & Bodine, J. H. (1942). *J. gen. Physiol.* **26**, 151.
Allen, T. H., Ray, O. M. & Bodine, J. H. (1938). *Proc. Soc. exp. Biol., N.Y.*, **39**, 549.
Arnon, D. I. (1949). *Plant Physiol.* **24**, 1.
Bodine, J. H. & Allen, T. H. (1938*a*). *J. cell. comp. Physiol.* **11**, 409.
Bodine, J. H. & Allen, T. H. (1938*b*). *J. cell. comp. Physiol.* **12**, 71.
Bodine, J. H. & Allen, T. H. (1941). *J. cell. comp. Physiol.* **18**, 151.
Bodine, J. H., Allen, T. H. & Boell, E. J. (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 450.
Bodine, J. H. & Boell, E. J. (1935). *J. cell. comp. Physiol.* **6**, 263.
Bodine, J. H. & Tahmisian, T. N. (1943). *Arch. Biochem.* **2**, 403.
Bodine, J. H., Tahmisian, T. N. & Hill, D. L. (1944). *Arch. Biochem.* **4**, 402.
Dawson, C. R. & Tarpley, W. B. (1951). *The Enzymes*, vol. 2, part 1, p. 471. New York: Academic Press.
Guggenheim, M. (1913). *Hoppe-Seyl. Z.* **88**, 276.
Harned, H. S. & Owen, B. B. (1939). *Chem. Rev.* **25**, 31.
Horowitz, N. H. & Fling, M. (1955). *Amino Acid Metabolism*, p. 207. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Horowitz, N. H. & Shen, S. C. (1952). *J. biol. Chem.* **197**, 513.
Kendal, L. P. (1949). *Biochem. J.* **44**, 442.
Kenten, R. H. (1955). *Abstr. 3rd Int. Congr. Biochem., Brussels*, p. 102.
Kenten, R. H. & Mann, P. J. G. (1952). *Biochem. J.* **50**, 360.
Mallette, M. F. (1950). *Copper Metabolism*, p. 49. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Ohnishi, E. (1954). *Annot. zool. jap.* **27**, 188. [Cited in *Chem. Abstr.* (1955). **49**, 7758 h.]
Paterson, J. S., Pirie, N. W. & Stableforth, A. W. (1947). *Brit. J. exp. Path.* **28**, 223.
Swartz, M. N., Kaplan, N. O. & Frech, M. E. (1956). *Science*, **123**, 50.

Sterol Metabolism

3. THE DISTRIBUTION AND TRANSPORT OF STEROLS ACROSS THE INTESTINAL MUCOSA OF THE GUINEA PIG*

By J. GLOVER and C. GREEN
Department of Biochemistry, The University of Liverpool

(*Received* 27 *December* 1956)

Animal tissues contain different proportions of several closely related sterols, and in the intestinal mucosa at least one of these, 7-dehydrocholesterol ($\Delta^{5:7}$-cholestadien-3β-ol), has been shown to be in equilibrium with the remainder (Glover, Glover & Morton, 1952). These sterols are composed mainly of cholesterol (Δ^5-cholestenol) and some lathosterol (Δ^7-cholestenol) (Moore & Baumann, 1952). It was of interest to make a more detailed study of intestinal mucosa to determine the proportion of 7-dehydrocholesterol in the various cell components; the sterols in some parts of the cell structure might not be exposed to the dehydrogenase enzymes, and hence the percentage of 7-dehydrocholesterol in the total present in those components would differ from the average value for the whole cell. A difference in the distribution of one sterol relative to another could help to establish the site of the enzyme concerned. Further information might also be obtained by observing changes in the sterol content of the mucosal-cell components while 7-dehydrocholesterol or cholesterol was being absorbed. Again, by direct examination of the fraction containing the fatty globules derived from the cell, it would be possible to test the hypothesis that the absorption of sterols was closely linked to that of fat, possibly by virtue of their solubility in it (Frazer, 1955).

Only a few studies have previously been carried out on the distribution of sterols in the cell components. Swanson & Artom (1950) demonstrated that lipid from rat-liver mitochondria contained 4·4% of cholesterol, which concentration is similar to that for the whole tissue. Chauveau, Clement, Clement-Champougny & Le Breton (1951) and recently Schotz, Rice & Alfin-Slater (1953) found cholesterol in all cell fractions obtained by differential centrifuging of liver cells. Even more recently Spiro & McKibbin (1956) have made a detailed study of the distribution of phospholipid and sterol in rat-liver cells. In our preliminary work liver cells of guinea pig were studied to check the fractionation technique, but the main investigations were concerned with the intestinal mucosa. While the present results have not clearly indicated the localization of the dehydrogenase enzyme, a better understanding of the absorption of sterols has been obtained. The preliminary findings have already been published (Glover & Green, 1954, 1956).

* Part 2: Glover, Leat & Morton (1957).

EXPERIMENTAL

Animals. Female guinea pigs, 220–550 g., supplied by an accredited breeder, were placed on our standard no. 4 cube diet (British Extracting Co. Ltd., Bromborough) with a supplement of cabbage leaves until the start of the experiment; 3–4 days before selection for test, they were placed on a sterol-free diet (Glover, Leat & Morton, 1957). Then they were grouped in pairs of uniform total weight and fasted for 24 hr. The control undosed groups were killed, after anaesthetization and withdrawal of blood by cardiac puncture, and examined directly. The test animals were each given 150 mg. of cholesterol or 7-dehydrocholesterol dispersed in 1 ml. of arachis oil mixed with 5 g. of the lipid-free diet. After 24 hr. another 150 mg. of the sterol was administered in the same way and the animals were left for 5–6 hr. before killing, as described above. By this procedure it was hoped that absorption would be occurring along the whole intestine at the time of examination. The livers and small intestines were excised and the latter washed out with 0·88 M-sucrose solution. All tissues were kept in ice-cold 0·88 M-sucrose solution until required.

Preparation of mucosal cells. The two small intestines, freed from mesenteric fat, were slit longitudinally and cut into short lengths which were spread flat on a cold glass surface (0–5°), i.e. the lid of a large Petri dish containing crushed ice and a little salt. The mucosal cells were scraped off with the blunt edge of a scalpel or spatula, collected in a small beaker (10 ml.), immersed in crushed ice, and then transferred completely to the homogenizer. Histological examination of tissue sections showed clearly that this treatment removed only the uppermost cells of the villi, i.e. the cells more exposed to food in the lumen and more active in lipid absorption (Hewitt, 1954). In later experiments, to sharpen further the differences between the fasting and dosed animals, only the mucosa from the proximal third of the intestine was examined because it is better developed in this portion and in fact contains almost half the total mucosal sterol (see Table 5).

The distribution of 7-dehydrocholesterol in relation to the total sterol is fairly uniform along the intestine of the fasting animal (Glover *et al.* 1952). This was confirmed by the present work (Table 5) so that no appreciable error was introduced by sampling only the proximal third. Histological examination of the residual tissue after scraping showed that the scrapings consist mainly of mucosal cells

with a small amount of the underlying connective tissue, but none of the main lymph vessels of the intestine was removed.

Preparation of homogenates. Liver tissue was cut into small pieces and placed in a porcelain mortar with cold 0·25 M-sucrose (1 vol.), and roughly broken up by pressure with a pestle. The resulting mince was filtered through 50-mesh nylon, diluted with 4 vol. of 0·25 M-sucrose and transferred to a Potter–Elvehjem homogenizer, which was previously chilled to 0°.

Movement of the homogenizer mortar (35 ml. capacity) up and down smoothly over the rapidly rotating (4000–5000 rev./min.) Teflon (polytetrafluoroethylene) pestle about four times for a total period of 1 min. sufficed to break up the greater portion of the cells.

The homogenate of combined mucosal cells was prepared in a similar manner, the final volumes usually being 30–40 ml. Suitable portions (2–5 ml.) for analysis were immediately withdrawn and the whole of the remainder was fractionated.

Separation of cell components. The fractionation procedure which follows that of Schneider & Hogeboom (1950) is shown in Fig. 1. Steps 1–3 were carried out in 15 ml. centrifuge tubes in a small centrifuge placed in a large refrigerator (−2°). The resulting 'cell-debris' fraction, containing unbroken cells and cell membranes in addition to the nuclei, was not fractionated further for the present work. The supernatants from the above steps were then mixed and transferred to 50 ml. plastic centrifuge tubes for the preparation of the mitochondria and microsome pellets. This section of the work was carried out in a high-speed angle-head centrifuge fitted with a refrigeration coil which maintained the temperature of the preparation between −3° and +5°. Before separation of the microsomes, however, a layer of light petroleum (b.p. 40–60°)–diethyl ether (1:1, v/v) was carefully placed on top of the suspension

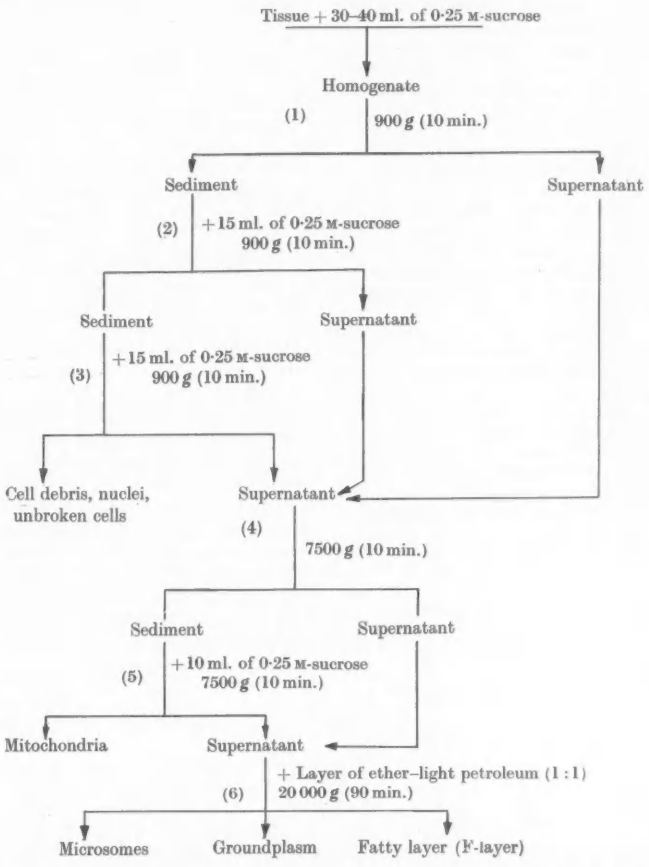


Fig. 1. Method of fractionating cell components by centrifuging the tissue homogenate in 0·25 M-sucrose at −3° to +5°.

(stage 6) so that, during the sedimentation process, fatty droplets migrating centripetally were collected in the organic solvent and not left sticking to the side of the centrifuge tube. A capillary pipette enabled the latter fraction (called F-layer) to be removed cleanly. The mitochondrial pellet, which had a pale-grey appearance, consisted of a fairly compact portion with a lighter fluffy layer on top. Microscopic examination of a dilute suspension showed that it was free from nuclei and broken cells but probably contained some large microsomes. The translucent microsome pellet was dull pink and very compact. The transparent pale yellow–brown supernatant (groundplasm) was easily separated by decantation. In view of the relatively low g value employed here compared with that in other Laboratories, it is probable that some of the very small microsomes (less than 50 mμ diam.) would be left in this fraction. These, however, have a much smaller amount of lipid than the larger ones (Barnum & Huseby, 1948).

Extraction of lipids. The various pellets were separately ground with anhydrous sodium sulphate and a little washed sand in a mortar; each mixture was transferred to a beaker and extracted four times with 50 ml. of boiling, dry peroxide-free ether. A further extraction with boiling ethanol did not remove any more sterol. The combined ether extracts were evaporated to dryness under nitrogen on a hot-water bath and the residues dissolved in spectroscopically pure ethanol for assay of the 7-dehydrocholesterol.

The groundplasm fraction was poured into sufficient ethanol to make the final alcohol concentration 70%, to denature the proteins. The lipids were then extracted twice with light petroleum (b.p. 40–60°), once with diethyl ether–light petroleum (1:1) and once with ether. These extracts were combined, washed twice with water and dried over anhydrous sodium sulphate. The solvent was evaporated off and the lipid residue dissolved in a suitable volume of spectroscopically pure ethanol (10–25 ml.).

Estimation of 7-dehydrocholesterol. The ultraviolet-absorption spectra of the above solutions suitably diluted were recorded over the range 230–350 mμ. The amount of 7-dehydrocholesterol present was then calculated from the extinction of the solution at 281·5 mμ after correction for irrelevant absorption (Glover *et al.* 1952).

Estimation of total sterol. A portion of the lipid solution in ethanol was taken to dryness, redissolved in chloroform and the sterol content determined by the method described by Kenny (1952), an EEL colorimeter being used. The range of sterol concentration for minimum error with this instrument was between 0·3 and 1·3 mg./10 ml. of final solution. For values below this range, the modification of the Kenny method as used by Green, Lowe & Morton (1955) was employed. In this procedure the extinction of the coloured solution at $\lambda_{max.}$ 410 mμ was read in a Unicam SP.600 photoelectric spectrophotometer.

Estimation of Δ^7-cholestenol (lathosterol). The colorimetric method, described by Moore & Baumann (1952) for total fast-reacting sterols (Δ^7-cholestenol and $\Delta^{5:7}$-cholestadienol), was followed except that the volumes of the reacting solutions were increased 1·5 times to suit the EEL colorimeter cells. Filter 607 was used.

Pure crystalline 7-dehydrocholesterol and cholesterol, purified via the dibromide process (Schwenk & Werthessen, 1952), and chromatography on alumina to remove traces of Δ^7-cholestenol contaminants, were employed for the standardization of the procedure.

The total content of fast-reacting sterols in the fraction minus that of 7-dehydrocholesterol gives the amount of lathosterol present.

RESULTS

Liver. The experiments with liver tissue were of a preliminary nature in order to evolve a suitable technique of cell fractionation which permitted good recovery of the sterols. In four experiments, the sterol distribution was found to be in general agreement with the results of Schotz *et al.* (1953) for rat liver. The recovery of sterol, however, was only 75–88% of the total in the homogenate. The losses were found to be in large part due to inadequate refrigeration during the fractionation, particularly in the high-speed centrifuge where the temperature of the material in the cups at the end of a 90 min. run rose to about 25–30°. For the work on the intestine, the refrigeration was improved so that the temperature of the tissues was kept between −3° and +5° and recoveries were much better (90–107%).

Intestine. The distribution of total sterol in the various fractions of intestinal-mucosal cells from fasting guinea pigs is indicated in Table 1. In those cases where total recovery was greater than the value obtained by analysis of the original homogenate, the higher figure was taken for the calculation of the percentage distribution.

Table 1. *Distribution of total sterols, mainly cholesterol, in the various cell fractions of intestinal mucosa of the fasting guinea pig*

Absolute amounts of sterol found in each fraction are given together with the mean values of the amounts expressed as percentages of the total sterols in the homogenate.

Fraction	Total sterol (mg.) I	II	III	IV	V	VI	VII	Mean ± S.D. (% of total)
Homogenate	7·65	7·32	24·1	17·64	29·2	9·13	10·23	—
Debris and nuclei	0·72	1·60	3·58	3·41	5·80	2·96	2·00	18·9 ± 2·8
Mitochondria	0·68	1·36	5·90	3·27	5·43	1·76	2·72	18·4 ± 5·7
Microsomes	4·04	4·40	13·75	5·73	9·77	3·76	4·08	43·2 ± 10·5
Groundplasm	0·76	0·54	4·44	2·59	8·40	0·68	0·80	13·3 ± 8·4
F-layer	0·09	0·20	0·04	0·02	0·10	—	—	0·9 ± 1·0
Recovery (%)	82·4	107·0	98·0	101·0	85·0	100·3	94·0	—

On the whole the distribution pattern is quite similar to that found for liver tissue. The greatest portion of the sterol in the cell is associated with the microsome fraction. It is of interest that an appreciable amount of the sterol is dispersed in the transparent groundplasm fraction, presumably conjugated with protein. Only a very small amount of cholesterol is present in the F-layer of the intestine compared with that from the liver. This is to be expected, since the latter fraction contains mainly cholesterol in the esterified form (Schotz *et al.* 1953) known to be absent from the intestine of the fasting guinea pig (Glover *et al.* 1952).

The results for animals absorbing cholesterol or 7-dehydrocholesterol are given in Table 2. They show little variation from the controls. In experiment VI the groundplasm and F-layer fractions were much higher than the others. In this instance the whole of the intestinal mucosa was taken, the fat content was higher and consequently may have affected the separation.

Relative distribution of the various sterols in mucosal-cell fractions. The ultraviolet-absorption spectra of the lipids from the various fractions except the F-layer of the fasting animal show clearly the characteristic absorption bands of 7-dehydrocholesterol (Fig. 2) in each. Whereas it is absent from the F-layer in the fasting animal it was

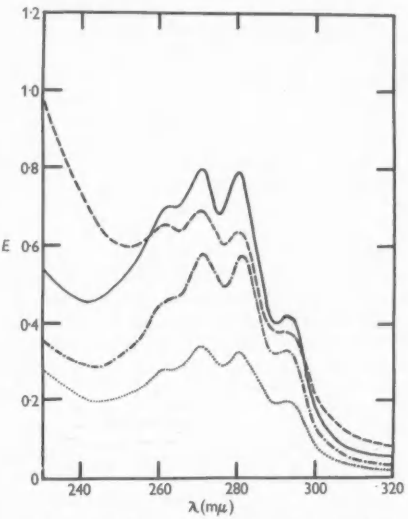


Fig. 2. Ultraviolet-absorption spectra of lipids from the cell components of intestinal mucosa fractionated at 0°. Cell debris and nuclei, –·–·; mitochondria, ……; microsomes ——; groundplasm, – – –.

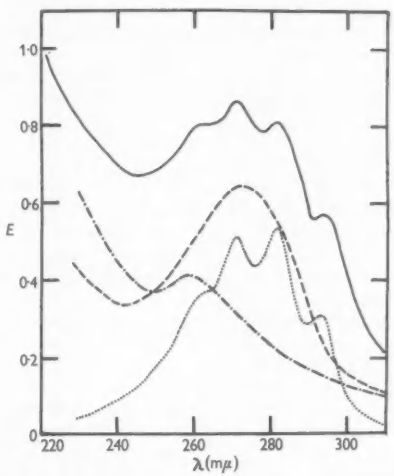


Fig. 3. Ultraviolet-absorption spectra of chromatographic fractions of lipids from mucosal-cell components fractionated at room temperature. Original lipids, ——; fraction *A*, – – –; fraction *B*, –·–·; 7-dehydrocholesterol, fraction *C*, …… For preparation of fractions see Results section.

Table 2. *Distribution of total sterols in the various cell fractions of intestinal mucosa of female guinea pigs dosed with 150 mg. each of cholesterol and 7-dehydrocholesterol*

Amount of total sterol in each cell component is expressed as a percentage of the total recovered. The total sterol (in mg.) in the original homogenate and the percentage recovered are also included.

	Cholesterol			7-Dehydrocholesterol		
Fraction	I	II	III	IV	V	VI
Homogenate (mg. of sterol)	30·0	11·0* (% of total)	32·9	10·3*	8·3* (% of total)	28·3
Debris and cell nuclei	31·3	18·9	24·6	21·3	29·7	12·6
Mitochondria	16·2	24·7	14·3	29·4	17·1	12·6
Microsomes	41·2	42·2	37·7	38·6	38·9	29·7
Groundplasm	10·0	12·2	23·4	10·7	14·3	33·7
F-layer	1·3	2·9	—	—	—	11·3
Recovery (%)	97	101	107	96	105	95

* Mucosa was taken only from proximal third of the intestine.

detected in that fraction from the animal dosed with 7-dehydrocholesterol.

In some of the preliminary experiments, marked destruction of 7-dehydrocholesterol occurred during the fractionation and subsequent isolation processes. The absorption spectra of fractions where the recovery of 7-dehydrocholesterol was poor are characterized by a greater extinction at 271·5 mμ than at the $\lambda_{max.}$ 281·5 mμ (Fig. 3). Since the absorption spectra of the tissue lipids dissolved in ethanol did not change appreciably when left in the dark at room temperature for 7 days, the change must have occurred during the cell-fractionation procedure. This was confirmed by leaving a mitochondrial preparation at room temperature for 2 hr. before extraction, when it lost about 30 % of its 7-dehydrocholesterol content. The separated lipoproteins containing the sterols are stable, on the other hand, for a few hours at room temperature. The change appears therefore to be enzymic since, by maintaining the preparations at less than 5° during the cell fractionation, 90–100 % recovery of this relatively labile sterol was always obtained.

When the contribution due to 7-dehydrocholesterol absorption is subtracted from the spectrum of the tissue lipids of mitochondria which have been kept at room temperature for a few hours, the residual absorption shows maxima at 260 and 272 mμ.

Chromatography of the lipids on alumina [weakened by adding 2 % (w/w) water] yielded, apart from known sterols, two impure fractions *A* and *B* (eluted with 4 % ethyl ether in light petroleum and 6 % ether in light petroleum respectively), each of which had a single maximum at 272 and 260 mμ respectively. The former chromophore was previously reported (Glover *et al.* 1952) in the same chromatographic fraction of guinea-pig intestinal lipids. The substance responsible was first obtained in purified form by Festenstein (1951), and has been designated SA by Festenstein, Heaton, Lowe & Morton (1955). It is not yet fully characterized. The substance giving rise to the chromophore in the second fraction is also unknown. A third fraction *C* (eluted with 8 % ether in light petroleum) contained most of the 7-dehydrocholesterol free from selectively absorbing constituents.

It is an odd feature, however, of the spectra of the mitochondria and microsome fractions that although the recovery of 7-dehydrocholesterol was excellent, often the extinction at 271·5 mμ is just higher than that at 281·5 mμ, although for the original homogenate it is invariably less. No satisfactory reason has yet been found for the increase in the irrelevant steeply rising end-absorption of the component lipids over that of the homogenate. It could possibly be due to a more efficient extraction of the lipids after the mechanical breakdown of the cells, or perhaps to some oxidative process during the cell fractionation.

Fasting animals. In seven experiments with animals maintained on the sterol-free diet for a few days before examination, the recoveries of 7-dehydrocholesterol from the cell fractions were satisfactory and the percentage in relation to total sterol in each was calculated. The results (Table 3) show that the distribution of the 7-dehydrocholesterol (which makes up about 9 % of the total sterol in each fraction) is very uniform across the cell.

Dosed animals. The results for the percentage of 7-dehydrocholesterol in the various mucosal-cell fractions from animals dosed with cholesterol and 7-dehydrocholesterol are set out in Table 4. The values for the former group are all lower and those for the latter all higher than the controls. Apparently, each fraction has taken up some of the administered sterol, causing a change in the relative amounts of the sterols present, but at the same time the distribution of the 7-dehydrocholesterol across the cell remains quite uniform. In Expts. II, IV and V only the proximal thirds of the intestines were examined. It was thought that by restricting the examination to the absorbing proximal third of the intestine, any difference between fasting and dosed animals would be accentuated. It is seen from Table 5, where the percentage of 7-dehydrocholesterol in the proximal

Table 3. *Distribution of 7-dehydrocholesterol in the cell fractions of intestinal mucosa of the fasting guinea pig*

Fraction	7-Dehydrocholesterol (% of total sterol) I	II	III	IV	V	VI	VII	Mean ± S.D.
Homogenate	8·7	9·4	9·2	9·9	8·5	9·3	—	9·2 ± 0·5
Debris and nuclei	11·3	11·5	8·4	9·4	8·8	8·6	10·1	9·7 ± 1·3
Mitochondria	6·2	8·9	8·5	6·5	10·4	10·4	10·4	8·8 ± 1·8
Microsomes	11·4	8·1	11·4	7·5	9·8	9·3	10·8	9·9 ± 1·3
Groundplasm	6·7	9·6	Very low	8·9	—	3·2	7·7	7·4 ± 2·8 (8·2 ± 1·3)*

* Mean value of the four highest results.

Table 4. *Distribution of 7-dehydrocholesterol in the cell fractions of intestinal mucosa of animals dosed with cholesterol (group A) and 7-dehydrocholesterol (group B)*

Dose was 150 mg. to each of the two animals per experiment.

	7-Dehydrocholesterol (% of total sterol)							
	Group *A*				Group *B*			
Fraction	I	II	III	Mean ± S.D.	IV	V	VI	Mean ± S.D.
Homogenate	6·6	6·8	7·3	6·9 ± 0·4	14·5	14·3	—	14·4 ± 0·1
Debris and nuclei	5·9	6·1	8·1	6·7 ± 1·2	12·7	14·9	18·9	15·5 ± 2·9
Mitochondria	5·3	7·0	7·2	6·5 ± 1·1	10·9	15·4	20·0	15·4 ± 4·5
Microsomes	7·6	6·7	7·6	7·3 ± 0·5	14·9	15·4	20·4	16·9 ± 3·1
Groundplasm	10·5	5·5	4·2	6·7 ± 3·3	13·2	11·6	13·3	12·7 ± 1·0
F-layer	—	—	—	—	—	—	9·4	—

Table 5. *Distribution of tissue sterols between the proximal third and distal two-thirds of the length of the small intestine of the guinea pig*

		7-Dehydrocholesterol	
Tissue section	Total sterol (mg.)	(mg.)	(% of total sterol)
Mucosa			
Proximal third	11·0	1·02	9·3
Distal two-thirds	13·0	1·03	7·9
Total	24·0	2·05	8·5
Muscle			
Proximal third	12·8	0·32	2·5
Distal two-thirds	16·0	0·38	2·4
Total	28·8	0·70	2·4
Whole intestine	52·8	2·75	5·2

Table 6. *Distribution of slow- and fast-reacting sterols in the cell fractions from intestinal mucosa*

Values were obtained by the method of Moore & Baumann (1952).

	Controls			Cholesterol fed			7-Dehydrocholesterol fed		
Fraction	Slow (mg.)	Fast (mg.)	Fast (%)	Slow (mg.)	Fast (mg.)	Fast (%)	Slow	Fast	Fast (%)
Homogenate	—	—	—	22·2	2·85	11·4	4·60	6·40	58·2
Debris and nuclei	5·98	1·81	23·2	7·1	0·98	12·1	0·68	1·00	59·5
Mitochondria	0·77	0·17	18·1	3·2	0·36	10·1	0·70	0·89	56·0
Microsomes	3·60	1·10	23·4	7·4	1·2	13·9	1·40	2·30	62·0
Groundplasm	0·42	0·10	19·2	2·0	0·31	13·4	1·50	1·50	50·0
F-layer	—	—	—	—	—	—	0·46	0·18	28·1

Table 7. *Distribution of lathosterol in cell fractions of the intestinal mucosa of the guinea pig*

Results, obtained by deducting the amount of 7-dehydrocholesterol from the total amount of fast-reacting sterols, are expressed as a percentage of total slow and fast.

Fraction	Controls	Cholesterol fed	7-Dehydrocholesterol fed
Homogenate	—	1·4	15·4
Debris and nuclei	10·1	2·8	21·4
Mitochondria	0·0	0·0	13·2
Microsomes	8·7	2·2	16·2
Groundplasm	5·8	0·0	9·7

third and the distal two-thirds mucosal fractions are compared, that the selection of the proximal third does not cause an appreciable change in the control value for the percentage concentration of 7-dehydrocholesterol.

The distribution of the total fast-reacting sterols, lathosterol and 7-dehydrocholesterol, was also studied in two experiments. In Table 6 are listed the percentages of fast- and slow-reacting sterols obtained for each fraction in mucosal cells from controls and animals given (*a*) cholesterol and (*b*) 7-dehydrocholesterol. Again, the distribution of the total fast-reacting sterols (relative to the slow

reacting) appears to be remarkably uniform, which supports the previous results. The percentages of lathosterol in the various mucosal fractions of the above animals are given in Table 7. The increase above the control level observed in the latter group indicates some conversion of the dietary 7-dehydrocholesterol into lathosterol in the mucosa.

DISCUSSION

The values for the overall distribution of cholesterol across the guinea-pig intestinal-mucosal cells (Table 1) show that the microsomes contain the greatest proportion and the mitochondria the next. These are in general agreement with the results for the liver cells and also with those obtained from rat liver (Schotz *et al.* 1953; Spiro & McKibbin, 1956). The cholesterol content of the F-layer from mucosal cells, however, is very small compared with that from liver cells, which contains mainly esterified cholesterol. The latter is only occasionally detectable in the mucosal fraction from fasting animals (unpublished observations). Since the sterols in the intestinal mucosa are unesterified (Glover *et al.* 1952) and not in solution in the fatty globules, they are presumably in association with other lipids in the formation of the different characteristic membrane-like structures similar to those observed histologically by Sjöstrand & Rhodin (1953) in mitochondria and groundplasm fractions of mouse-kidney-tubular cells and in other tissue cells.

With regard to the uniform distribution of 7-dehydrocholesterol relative to total sterols between the cell fractions, the results in Table 3 show firstly that there is no preferential localization of one sterol in a particular fraction. Hence the location of the dehydrogenase enzyme, which brings about the formation of the related sterols, cannot yet be determined by this procedure. Secondly, they indicate a rapid turnover or exchange of the greater part of the various sterols between their lipoprotein components. Support for this view comes from the fact that in the dosed animal also, despite the change in the percentage content of 7-dehydrocholesterol in the total sterols of each fraction, the values for the different cell fractions still remain at a uniform level. In fact, this exchange or transfer process must take place in the cell faster than the oxidative–reductive steps responsible for maintaining the normal equilibrium between the sterols; otherwise it would not have been possible to demonstrate a change from the fasting level to the new values obtained in the dosed animals. Again, during the absorption of 7-dehydrocholesterol, Δ^7-cholestenol was formed and was found in uniform relation to the other sterols present. This finding has recently been confirmed in the rat (Glover & Stainer, 1956).

Gould (1951) has previously observed a rapid equilibration between plasma and blood cells of unesterified sterol attached to protein. Again, Gould (1955) found that the free cholesterol of plasma equilibrated much faster with the free cholesterol of liver than did the corresponding esters. Thus the exchange of the sterol between lipoproteins is faster than that promoted by cholesterol esterase between the esterified and free cholesterol. Phospholipids attached to protein have also been shown to exchange quite rapidly (Hahn & Hevesy, 1939). The above findings show that dietary sterol on absorption is taken out of the oily medium, in which it is administered, by the lipoproteins of the organelles and groundplasm and rapidly exchanged between them; only a relatively small amount of the sterol appears in the fatty globules dispersed within the cytoplasm. This leads to the conclusion that the lipid must be dispersed to the molecular level during the absorption process. It is also significant that esterified cholesterol must be hydrolysed to the free alcohol form before entry into the cell (Kim & Ivy, 1952; Favarger & Metzger, 1952). Further experiments on the form of cholesterol within the cell during absorption will be discussed more fully in a later paper.

It would appear therefore that the sterols are transported across the mucosal cell by means of this exchange between lipoproteins rather than by their migration in fatty globules. The latter are apparently temporary reservoirs of fat rather than carriers of dissolved sterol, the main absorption stream occurring at the molecular level on lipo proteins.

Whereas it has been established since the work of Mueller (1916) that dietary cholesterol appears in the lymph in large part esterified, and that fatty acids, particularly oleic acid, aid sterol absorption (Kim & Ivy, 1952), the main role of fatty acids seems to be concerned with the acceleration of sterol transfer from the mucosal cell into the lacteals. Esterification, however, does not appear to be the main controlling factor since *epi*cholesterol is absorbed unesterified (Hernandez, Chaikoff, Dauben & Abraham, 1954). Again, dihydrocholesterol, which is less-well absorbed than cholesterol (Gould, Lotz & Lilly, 1956), is more readily esterified than the latter (Swell, Field & Treadwell, 1955). Moreover, in work in this Laboratory to be described in later papers, the above exchange and transfer process has been confirmed with [^{14}C]cholesterol *in vivo* and *in vitro*. Furthermore, the various lipoproteins from the particulate fractions concerned have been resolved by paper electrophoresis and shown to carry the dietary sterols (Green, 1955).

With regard to the mechanism of the absorption process the transfer of the sterol across the cell must

involve the lipoproteins of the various cell fractions and is probably mediated by its adsorption and desorption on them in the same manner in which the endogenously synthesized sterols become associated with the various cell proteins. It would seem reasonable that sterol presented to the external side of the mucosal-cell membrane would exchange or readily displace a similar molecule within the membrane and hence penetrate the cell. Then further transfer of the sterol to the other lipoproteins could occur whereby the molecules would be passed across the mucosal cells to the connective tissue containing the lymph capillaries. Esterification of the sterol may then occur to hasten the transfer into the lacteals.

The finding of Biggs, Friedman & Byers (1951), that the specific activity of [^{14}C]cholesterol administered orally to rats is diluted approximately tenfold with endogenous sterol by the time it reaches the intestinal lymph, lends strong support to the mechanism proposed above.

Furthermore, the above concept of sterol absorption enables a satisfactory explanation of the poor absorption of phytosterols to be suggested. The tendency of the intestinal mucosa to reject the latter, which are only slightly different from cholesterol structurally, implies that stereochemistry is the main issue and not solubility in other lipids or chemical reactivity. It would seem that the phytosterols do not fit on to the acceptor lipoproteins so readily as the natural animal sterols and that they enter into exchanges more slowly than the latter, hence their transfer across the mucosal cells becomes inhibited.

The inhibition of cholesterol absorption by phytosterols, initially observed by Peterson (1951), could also be explained by assuming that the acceptor lipoprotein becomes partially blocked with the foreign sterol, thereby reducing its capacity for the transfer of the natural animal sterol.

SUMMARY

1. The distribution of total sterols and 7-dehydrocholesterol among the components of intestinal-mucosal cells of young fasting female guinea pigs and animals actively absorbing cholesterol and 7-dehydrocholesterol has been studied.

2. In the fasting animal, the sterols are associated with each of the fractions examined: cell membranes and nuclei, mitochondria, microsomes, groundplasm and fatty globules (F-layer). The microsomes contained about 40 % of the total in the cell, the mitochondria about 20 % with the remainder distributed equally between the cell membranes and nuclei fraction and the groundplasm. The amount in the F-layer was less than 0·5 %.

3. The sterols undergoing absorption were observed to enter each fraction but the number of results obtained was insufficient to determine unequivocally if the uptake represented an absolute increase in each fraction or merely an exchange between the dietary and endogenous molecules.

4. The distribution of 7-dehydrocholesterol in relation to total sterols and of fast-reacting to slow-reacting sterols was uniform across the mucosal cells of the fasting animals and tended towards uniformity in the dosed animals. These findings imply a rapid exchange of the dietary with the endogenous sterols during the passage of the former across the intestinal mucosa.

5. The above evidence leads to the conclusion that the absorption of sterols takes place at the molecular level via a rapid exchange and transfer process between the various lipoproteins of the cell membrane, organelles and groundplasm.

6. An explanation for the poor absorption of phytosterols and their inhibition of cholesterol absorption in relation to the above hypothesis is discussed.

7. Evidence was obtained for the reduction of 7-dehydrocholesterol to lathosterol during the absorption of the former across the intestine.

We wish to thank Dr N. M. Hancox, Histology Department, University of Liverpool, for examining the specimens of intestinal tissue. We also wish to thank the Medical Research Council for a grant towards the work. One of us (C. G.) was in receipt of a Medical Research Council Scholarship.

REFERENCES

Barnum, C. P. & Huseby, R. H. (1948). *Arch. Biochem. Biophys.* **19**, 17.

Biggs, M. W., Friedman, M. & Byers, S. O. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 641.

Chauveau, J., Clement, G., Clement-Champougny, J. & Le Breton, E. (1951). *Arch. Sci. Physiol.* **5**, 305.

Favarger, P. & Metzger, E. F. (1952). *Helv. chim. acta*, **35**, 1811.

Festenstein, G. N. (1951). Ph.D. Thesis: University of Liverpool.

Festenstein, G. N., Heaton, F. W., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **59**, 1955.

Frazer, A. C. (1955). *Nature, Lond.*, **175**, 491.

Glover, J. & Green, C. (1954). *Biochem. J.* **58**, xviii.

Glover, J. & Green, C. (1956). *Biochemical Problems of Lipids*, p. 359. Ed. by Popják, G. & Le Breton, E. London: Butterworths.

Glover, J., Leat, W. M. F. & Morton, R. A. (1957). *Biochem. J.* **66**, 214.

Glover, J. & Stainer, D. (1956). *Biochem. J.* **64**, 15P.

Glover, M., Glover, J. & Morton, R. A. (1952). *Biochem. J.* **51**, 1.

Gould, R. G. (1951). *Amer. J. Med.* **11**, 209.

Gould, R. G. (1955). *Résumés des Communications*, 3rd *Int. Congr. Biochem., Brussels*, p. 49.

Gould, R. G., Lotz, L. V. & Lilly, E. M. (1956). *Biochemical Problems of Lipids*, p. 353. Ed. by Popják, G. & Le Breton, F. London: Butterworths.
Green, B., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **61**, 447.
Green, C. (1955). Ph.D. Thesis: University of Liverpool.
Hahn, L. & Hevesy, G. (1939). *Nature, Lond.*, **144**, 72.
Hernandez, H. H., Chaikoff, I. L., Dauben, W. G. & Abraham, S. (1954). *J. biol. Chem.* **206**, 757.
Hewitt, W. (1954). *Quart. J. Micr. Sci.* **95**, 153.
Kenny, A. P. (1952). *Biochem. J.* **52**, 611.
Kim, K. S. & Ivy, I. C. (1952). *Amer. J. Physiol.* **171**, 302.
Moore, P. R. & Baumann, C. A. (1952). *J. biol. Chem.* **195**, 615.
Mueller, J. H. (1916). *J. biol. Chem.* **27**, 463.
Peterson, D. W. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 143.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Schotz, M. C., Rice, L. I. & Alfin-Slater, R. B. (1953). *J. biol. Chem.* **204**, 19.
Schwenk, E. & Werthessen, N. T. (1952). *Arch. Biochem. Biophys.* **40**, 334.
Sjöstrand, F. S. & Rhodin, J. (1953). *Exp. Cell Res.* **4**, 426.
Spiro, M. J. & McKibbin, J. M. (1956). *J. biol. Chem.* **219**, 643.
Swanson, M. A. & Artom, C. (1950). *J. biol. Chem.* **187**, 281.
Swell, L., Field, H. & Treadwell, C. R. (1955). *Proc. Soc. Exp. Biol., N.Y.*, **87**, 216.

The Mechanism of the Irreversible Inhibition of Rat-Liver Monoamine Oxidase by Iproniazid (Marsilid)

By A. N. DAVISON*
Roche Products Ltd., Broadwater Road, Welwyn Garden City, Herts

(*Received* 21 *November* 1956)

The physiological role of monoamine oxidase in the oxidation of adrenaline, 5-hydroxytryptamine and related amines is still the subject of controversy (Furchgott, 1955). Many investigators have used monoamine oxidase inhibitors such as choline *p*-tolyl ether, and particularly iproniazid (1-*iso*nicotinoyl-2-*iso*propylhydrazine phosphate, Marsilid), as tools in studies of this important problem (e.g. Zeller, Barsky & Berman, 1955*a*; Schayer, Smiley & Kaplan, 1952; Brodie, Pletscher & Shore, 1956; Griesmer & Wells, 1956). Despite the widespread use of iproniazid as an irreversible inhibitor, both *in vivo* and *in vitro*, little is known about the mechanism of the inhibition reaction.

In this paper the original observations of Zeller *et al.* (1955*a*), showing that pre-incubation of liver monoamine oxidase with iproniazid is necessary to obtain maximum inhibition, have been extended. Inhibition is easily prevented by the addition of substrate. In the absence of substrate, the irreversible inhibition reaction was found to be progressive and first-order, to require oxygen, and to have a high apparent energy of activation. These findings suggest that iproniazid reacts chemically at or near the enzyme active centre, and a hypothesis is advanced which accounts not only for this possibility but also for the structural requirements for inhibitors as shown by Zeller, Barsky, Fouts & Lazanas (1955*b*).

* Present address: Department of Pathology, Guy's Hospital Medical School, London, S.E. 1.

EXPERIMENTAL

Monoamine oxidase determinations. Livers from adult albino rats were chilled with ice, washed and suspended in Sørensen's 0·067 M-phosphate buffer, pH 7·2 (20 %, w/v), by means of a Potter–Elvehjem glass homogenizer. The suspension was centrifuged at room temperature for 2 min. at the maximum speed (approx. 4000 *g*) of a bench centrifuge (Baird and Tatlock Ltd.) and the resultant supernatant was then recentrifuged at 8500 *g* for 10 min. at 10° in a refrigerated centrifuge. The mitochondria so obtained were resuspended in the 0·067 M-phosphate buffer [equivalent to 300 mg. (wet wt.) of liver/ml.]. The enzyme activity was determined on 1 ml. of mitochondria suspension by using tyramine (0·01 M final concentration) as a substrate and adjusting the volume in the flasks to 4 ml. with buffer. Substrate and inhibitors were dissolved in the phosphate buffer. The flasks were flushed with oxygen before equilibration (total time 5 min.) and addition of the substrate. The bath temperature was 37°. Potassium hydroxide was not present in the centre well.

Kinetic measurements. The methods were similar to those of Davison (1956). The standard technique was to place 0·5 ml. of inhibitor solution and 0·25 ml. of tyramine solution in side arms of double-armed flasks and, after flushing with oxygen and equilibrating (5 min.), to add inhibitor from the side arm at different times. In these experiments the flasks containing enzyme were shaken at 37° for the same time and only the time of addition of inhibitor was varied. At the end of the incubation the substrate was added to all the flasks and manometer readings were taken every 5 min. for 35 min. The mean oxygen uptake was calculated by the method of least squares (Aldridge, Berry & Davies, 1949). Residual enzyme activity was expressed as a percentage of a control suspension

without added inhibitor. Oxygen uptake was found to be directly proportional to ammonia liberation.

Inhibitors. *iso*Propylhydrazine was prepared by refluxing 10 g. of iproniazid, 10 ml. of conc. HCl and 10 ml. of water for 1 hr. The mixture was steam-distilled. The distillate was neutralized with HCl and evaporated to dryness; the *iso*propylhydrazine hydrochloride, crystallized from an ethanol–ether mixture and recrystallized from dry acetonitrile, had m.p. 114° (cf. Lochte, Noyes & Bailey, 1922) (Found: C, 32·6; H, 9·84; N, 26·93; Cl, 31·8. $C_3H_{11}N_2Cl$ requires C, 32·7; H, 10·0; N, 25·5; Cl, 31·8%). 1-*iso*Propylidene-2-*iso*nicotinoylhydrazine was obtained from Roche Products Ltd.

RESULTS

Iproniazid and tyramine in various concentrations were added together to mitochondria. The reciprocal of the rate of oxygen uptake was plotted against the reciprocal of the tyramine concentration for each concentration of inhibitor and the dissociation constant for the iproniazid–enzyme complex calculated by the method of Lineweaver & Burk (1934). Under these conditions iproniazid was found to be a competitive inhibitor of monoamine oxidase (Table 1).

Pre-incubation of iproniazid at 37° in oxygen with the enzyme resulted in progressive and irreversible inhibition (Zeller *et al.* 1955*a*, and Table 4). Addition of high concentrations of substrate (0·01 M) stopped further inhibition, thus allowing activity determinations to be made. It was therefore possible to investigate the kinetics of inhibition by incubating iproniazid for different times at 37° with mitochondria before adding substrate and determining the residual enzyme activity. The kinetics of the reaction showed the characteristics of a bimolecular reaction with one component, presumably the inhibitor, in excess and obeyed the first-order equation (Fig. 1). The rate constant and values for 50% inhibition in 30 min. are given in Table 1. Since temperature had a pronounced effect on inhibition by iproniazid, the rates of inhibition at 22°, 30°, 37° and 45° were determined and the apparent energies of activation calculated from the Arrhenius equation (Table 1). High values were obtained for both the oxidation of tyramine and the inhibition reaction. These experiments suggested that irreversible inhibition of monoamine oxidase by iproniazid was the result of a chemical reaction probably at the enzyme active centre.

Progressive and irreversible inhibition did not occur if iproniazid was added to mitochondria under anaerobic conditions. Thus in one experiment Warburg flasks containing mitochondria with iproniazid in the side arm were gassed with nitrogen for 5 min. at 37°. The iproniazid (final concentration 10^{-4} M) was then tipped from the side arm and incubated with the mitochondrial suspension for 30 min. Tyramine was then added and the flasks were flushed with oxygen for 2 min. No inhibition of enzyme activity resulted, although in a control experiment with oxygen present throughout nearly complete inhibition was obtained.

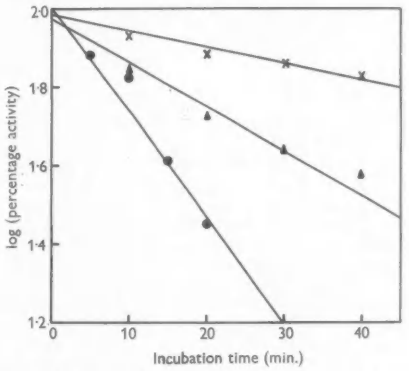


Fig. 1. Inhibition of monoamine oxidase activity by iproniazid. For details see the text. ×, Iproniazid, 6·6 μM; ▲, 20 μM; and ●, 100 μM.

Table 1. *Constants for inhibition of monoamine oxidase*

The constants have been determined with mitochondria as a source of monoamine oxidase and tyramine as substrate. For details see text.

Constant	Substrate (tyramine)	Inhibitors	
		Iproniazid	*iso*Propylhydrazine
Dissociation constant of inhibitor and substrate–enzyme complex determined by the Lineweaver & Burk (1934) method	3×10^{-3}	2×10^{-4}	3×10^{-5}
Approximate apparent energies of activation for the inhibitory reaction (cal./mole.)	17 000	30 000	26 000
Rate of reaction of inhibitor and enzyme at 37°, pH 7·2, (l. mole.$^{-1}$ min.$^{-1}$)	—	$1{\cdot}65 \times 10^3$	$1{\cdot}55 \times 10^4$
Concn. producing 50% inhibition at 37°, pH 7·2, on incubation for 30 min. (M)	—	$1{\cdot}4 \times 10^{-5}$	$1{\cdot}5 \times 10^{-6}$

Progressive inhibition of enzyme activity by iproniazid was found to be less for liver suspensions (25 %, w/v, in 0·067 M-phosphate buffer prepared as described above) than for mitochondrial preparations. This suggested that the supernatant was responsible for the insensitivity. It was found that if glutathione (0·8 mM) was added to mitochondria, the sensitivity was decreased and the inhibition was similar to that found on addition of the supernatant obtained by centrifuging an equivalent amount of liver suspension. Glutathione by itself had no effect on monoamine oxidase activity.

It has been shown that oxygen and iproniazid are necessary for irreversible inhibition of monoamine oxidase and that inhibition can be blocked by addition of substrate. Further, shaking iproniazid with mitochondria in an atmosphere of nitrogen does not result in inhibition of monoamine oxidase activity. It therefore seemed possible that iproniazid acted initially as a substrate for monoamine oxidase itself. This possibility is supported by the finding that the susceptibility of monoamine oxidase to inhibition by iproniazid remains the same after partial inactivation of the enzyme by heat treatment (Table 2). Similarly, inhibition of monoamine oxidase activity by iproniazid is affected by pH in the same way as tyramine oxidation (Fig. 2).

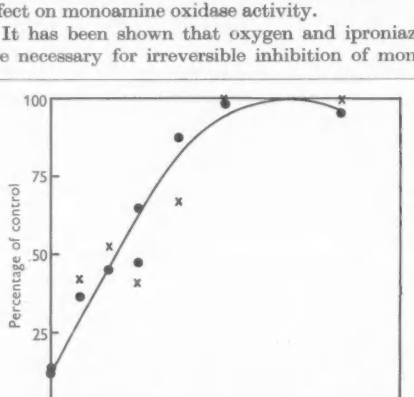


Fig. 2. Effect of pH on tyramine oxidation (●) and inhibition (×) by iproniazid of mitochondrial monoamine oxidase. The rate of oxidation of tyramine (0·01 M) by mitochondria (300 mg. wet wt. of liver) in Clark & Lub's borate (pH 10), Sørensen's (pH 6–8) and Walpole's acetate (pH 5) buffers was determined. 100 % activity was obtained at pH 8. Mitochondrial suspensions (0·5 ml., equivalent to 600 mg. wet wt. of liver) were incubated with iproniazid (0·1 mM) for 20 min. at 37° in each of the buffers (3 ml.). After incubation, 3 ml. of ice-cold saline was added to the Warburg flasks and the contents were centrifuged at 0°. The precipitated mitochondria were resuspended in 6 ml. of phosphate buffer, pH 7·2, and 3 ml. was taken for enzyme-activity determinations. Uninhibited controls were run. 100 % inhibition was taken as the inhibition obtained at pH 8.

Possible conversion of iproniazid into iso*propylhydrazine.* Since alkylhydrazines have been shown to be powerful inhibitors of monoamine oxidase (Zeller *et al.* 1955*b*) another possibility was that iproniazid was converted into the potentially much more reactive *iso*propylhydrazine before inhibition of monoamine oxidase could occur. *iso*Propylhydrazine was found to be an even stronger competitive inhibitor of monoamine oxidase than iproniazid (Table 1).

A study of the kinetics of inhibition of monoamine oxidase revealed that *iso*propylhydrazine, like iproniazid, progressively and irreversibly inhibited the enzyme when incubated in oxygen, but not in nitrogen. The rate of the inhibition reaction was calculated at different temperatures and the value of the apparent energy of activation was similar to that of iproniazid (Table 1). It therefore seemed unlikely that irreversible inhibition by iproniazid was due to prior formation of *iso*propylhydrazine. Additional evidence has been obtained in support of this view.

The reactivation of monoamine oxidase after inhibition by various substituted hydrazines was examined. In these experiments 1-*iso*nicotinoyl-2-methylhydrazine and methylhydrazine sulphate were also used, since both compounds are powerful monoamine oxidase inhibitors and resemble *iso*propylhydrazine and iproniazid in possessing a terminal alkylhydrazine group. Mitochondria, prepared in 0·25 M-sucrose, were incubated with the inhibitors for 30 min. at 37°, washed and reacti-

Table 2. *Effect of heat on inhibition of monoamine oxidase by iproniazid*

Suspensions of liver mitochondria (250 mg./ml. wet wt. of liver) in 0·067 M-phosphate buffer, pH 7·2, were heated for 10 min. at different temperatures. A portion (1 ml.) of each sample was incubated for 30 min. at 37° with 6·6 μM-iproniazid and tyramine oxidase activity determined as a percentage of an uninhibited control. Values given are the mean of two experiments.

Temp.	37°	47·5°	50°	52·5°	55°	60°
Activity of suspension without inhibitor (% of activity at 37°)	100	79	53	44	35	2C
Activity after treatment with iproniazid (% of uninhibited activity)	67	61	50	65·5	52	35·5

vated either by dialysis or by addition of dimercaptopropanol. Table 3 shows that although some return of monoamine oxidase activity was obtained after inhibition by iproniazid and 1-*iso*nicotinoyl-2-methylhydrazine, there was much less reactivation after inhibition by methyl- and *iso*propyl-hydrazines. Therefore the inhibition of the monoamine oxidase by the alkylhydrazines appears to differ in nature from that brought about by the two *iso*-nicotinoyl derivatives.

Inhibition of mitochondrial monoamine oxidase by iproniazid in the presence of cyanide. When iproniazid was added directly to mitochondria in the presence of cyanide and the flasks were incubated at varying times before addition of substrate, the inhibition obtained was progressive and the reaction was first-order (Fig. 3). The inhibition was also greater than that obtained in the absence of cyanide. Thus in the presence of cyanide mitochondrial monoamine oxidase activity was inhibited 81% by 20 μM iproniazid (20 min. at 37°), whereas in the absence of cyanide only 28% of enzyme activity was inhibited. Monoamine oxidase activity could not be restored by diluting either of the two inhibited preparations with ice-cold saline plus tyramine, centrifuging down the mitochondria at 0° and resuspending the mitochondria before determining their tyramine oxidase activity compared with uninhibited mitochondria similarly treated.

The possible reversibility of inhibition of mitochondrial monoamine oxidase by iproniazid in the presence of cyanide was also examined by a dilution technique (Aldridge, 1950). This technique depends on incubating a concentrated enzyme preparation with inhibitor and then diluting with buffer and substrate, or with buffer, substrate and inhibitor of the initial concentration (the substrate prevents further progressive inhibition). Table 4 (Expts. 1 and 2) shows that in the absence of cyanide the inhibition by iproniazid was not reversed on dilution. In the presence of cyanide, however, and under the conditions of this experiment, the additional inhibition obtained can be reversed by dilution (Expts. 3 and 4). Under anaerobic conditions in the presence of cyanide, inhibition by iproniazid can be completely reversed on dilution (Expts. 5 and 6).

In other experiments cyanide was placed with phosphate buffer and mitochondria in the centre compartment with tyramine and iproniazid in the

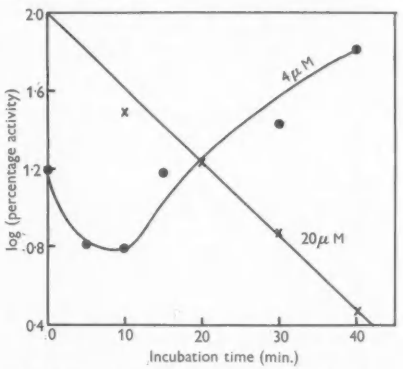


Fig. 3. Effect of cyanide on inhibition of monoamine oxidase by iproniazid. Each flask contained 1 ml. of mitochondrial suspension (equivalent to 300 mg. wet wt. of liver), KCN (0·002 M) and 0·067 M-phosphate buffer, pH 7·2, to give a final volume of 4 ml. Tyramine (0·01 M final concn.) and iproniazid (4 or 20 μM final concn.) were in each side arm. The incubation time represents the time of incubation of iproniazid with the enzyme. ×, Iproniazid was added immediately to the enzyme; ●, after 0, 10, 25, 30, 35 and 40 min.

Table 3. *Reaction of mitochondrial monoamine oxidase after inhibition by substituted hydrazines*

A suspension of mitochondria was prepared in cold 0·25 M-sucrose from rat liver (500 mg. wet wt. of original liver/ml.). Suitable volumes were inhibited by 5 mM-iproniazid, mM-*iso*propylhydrazine, mM-methylhydrazine, and 2 mM 1-*iso*nicotinoyl-2-methylhydrazine for 30 min. at 37°. The mitochondria were resedimented at 8000 *g* at 0° and resuspended in Sørensen's 0·067 M-phosphate buffer, pH 7·2, to give their original volume. After dialysis the enzyme activity was determined directly; after treatment with dimercaptopropanol (BAL) the mitochondria were centrifuged down and resuspended. Inhibited and uninhibited controls were run in all cases. The numbers of experiments are shown in brackets and mean values given. In the dialysis experiments two series of experiments were run as shown.

Monoamine oxidase inhibited by	Reactivation (%): Dialysis of 5 ml. of mitochondrial suspension for 18 hr. at 20° against 2 l. of mM Sørensen's phosphate buffer, pH 7·2		BAL, final concn. 0·01 M
Iproniazid	53 (2)	10 (2)	23 (3)
*iso*Propylhydrazine hydrochloride	16 (2)	4 (2)	1 (3)
1-*iso*Nicotinoyl-2-methylhydrazine hydrochloride	45	10	24
Methylhydrazine sulphate	10	0	2

Table 4. *Reversibility of inhibition of monoamine oxidase of mitochondria*

Mitochondrial suspensions (0·5 ml., equivalent to 300 mg. of liver/ml.) were incubated with 50 μM-iproniazid at 37° in O_2 or N_2 (Expts. 5 and 6) in the side arm of a double-armed Warburg flask. In Expts. 3–6 KCN (0·002 M) was present (in both side arm and central compartment). After 5, 10, 20 and 40 min. incubation the mitochondria were tipped into the central compartment containing eight times its volume of either buffer containing tyramine (0·01 M) or buffer containing tyramine plus iproniazid (50 μM). Where nitrogen was used, the flasks were then flushed with oxygen before addition of substrate. After addition of tyramine the oxygen uptake was determined and the enzyme activities were calculated as a percentage of uninhibited controls.

Enzyme activity on dilution into buffer with tyramine (0·01 M) plus	Enzyme activity (%) after incubation of iproniazid and enzyme for			
	5 min.	10 min.	20 min.	40 min.
1. Iproniazid	71·5	74	51	21
2. —	78·5	78·5	63·5	23
3. Cyanide	78	71	55	20
4. Cyanide + iproniazid	13	>5	>5	20
5. Cyanide (shaken in N_2)	100	100	100	100
6. Cyanide + iproniazid (shaken in N_2)	27	54	60	52

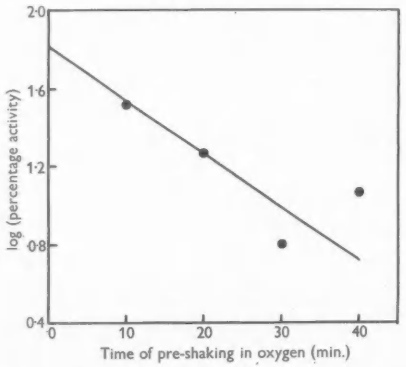


Fig. 4. Effect of shaking mitochondrial monoamine oxidase in oxygen with cyanide on inhibition by iproniazid. Each flask contained 1 ml. of mitochondrial suspension (equivalent to 300 mg. wet wt. of liver). Tyramine (0·01 M) and iproniazid (4 μM) were present in the side arms and KCN (0·002 M) and 0·067 M-phosphate buffer, pH 7·2, in the central compartment to give a final volume of 4 ml. After gassing with oxygen the enzyme preparation was shaken for various times before incubation with iproniazid for 10 min. and determining the resultant inhibition.

side arms. After gassing with oxygen, the inhibitor was added to the centre compartment at different times (0, 10, 20, 30 and 40 min.) and then tyramine added for enzyme-activity determination. In this way the monoamine oxidase preparation was shaken for a constant 40 min. with cyanide and buffer but for different times of incubation with the inhibitor. Under these conditions, instead of progressive inhibition, there was an immediate and greatly increased inhibition which apparently became less with increased time of incubation of enzyme and iproniazid (Fig. 3). The inhibition could not be reversed by washing the inhibited mitochondria. Shaking the inhibitor in phosphate buffer with or without 0·002 M-potassium cyanide had no influence on the degree of inhibition obtained with an unshaken mitochondrial preparation. Fig. 4 shows the effect of shaking liver mitochondria, in oxygen and with cyanide, for different times in Warburg flasks before addition of iproniazid to the enzyme, incubating for 10 min. and subsequently determining enzyme activity. It will be noted that the longer the pre-shaking of the mitochondria in oxygen with cyanide the greater was the inhibition produced by the iproniazid. This sensitizing effect did not increase after 50–60 min. It seemed, therefore, that pre-shaking liver mitochondria under these conditions increased the susceptibility of the monoamine oxidase to inhibition by iproniazid. This was substantiated by determining the approximate dissociation constant of the enzyme inhibitor complex for mitochondria under different conditions of pretreatment. The dissociation constant for the combination of iproniazid and monoamine oxidase in mitochondria was not changed by prior shaking in nitrogen or oxygen. But in the presence of cyanide the approximate affinity constant (1/dissociation constant) increased a hundred times on prior shaking with oxygen for 30 min. before addition of iproniazid and tyramine (Table 5).

Rat-liver mitochondria were shaken in oxygen, at 37° with potassium cyanide for 50 min., to sensitize them fully to inhibition by iproniazid. The kinetics of inhibition of this enzyme preparation by iproniazid (1 μM) were examined. Inhibition was immediate, not progressive and irreversible. The same sensitized mitochondria were diluted with saline, centrifuged down and resuspended in phosphate buffer. No inhibition could be obtained on incubating with iproniazid (4 μM) for 10 min. Thus sensitization by cyanide can be readily reversed by washing.

Table 5. *Effect of pre-shaking in oxygen and nitrogen on the affinity of iproniazid for mitochondrial monoamine oxidase*

Mitochondrial suspensions (1 ml., equivalent to 300 mg. wet wt. of liver/ml.) in phosphate buffer were placed in the central compartment of Warburg flasks with or without KCN (0·002 M). Suitable concentrations of tyramine and iproniazid were placed in the side arms. Sufficient phosphate buffer was added to give a final volume of 4 ml. The approximate inhibitor dissociation constant was calculated by the Lineweaver & Burk (1934) method.

	Approximate dissociation constant calc. after pre-shaking in		
Prep.	Nitrogen (10 min.)	Oxygen (5 min.)	Oxygen (30 min.)
No KCN	1×10^{-4}	2×10^{-4}	2×10^{-4}
KCN	6×10^{-5}	6×10^{-6}	5×10^{-7}

Preliminary experiments indicate that inhibition of monoamine oxidase by *iso*propylhydrazine can also be increased by adding cyanide.

Possible dehydrogenation of iproniazid as a prerequisite for inhibition. It has been shown that oxidation of iproniazid is necessary for irreversible inhibition of monoamine oxidase, and it therefore seemed possible that iproniazid acted initially as a substrate for the enzyme. It has been suggested (Blaschko, 1952) that amines are first dehydrogenated to give an imino compound; thus dehydrogenation of iproniazid to 1-*iso*propylidene-2-*iso*nicotinoylhydrazine would be a necessary step in forming an irreversible inhibitor. 1-*iso*Propylidene-2-*iso*nicotinoylhydrazine, however, did not inhibit monoamine oxidase at mM *in vitro* or in mice *in vivo*. Mice were given 250 mg. of 1-*iso*propylidene-2-*iso*nicotinoylhydrazine/kg. by intraperitoneal injection. After 1 and 5 hr. pairs of animals were killed. Liver suspensions (25 %, w/v) were prepared in phosphate buffer and monoamine oxidase activity was determined on 1 ml. with tyramine (0·01 M) as substrate. There was no inhibition of enzyme activity when compared with liver preparations from control animals. It is also of interest that isoprenaline sulphate [1-(3:4-dihydroxyphenyl)-2-*iso*propylaminoethanol sulphate], which possesses the $\cdot NH \cdot CH(CH_3)_2$ terminal group, at a concentration of 0·01 M had little effect on the rate of tyramine oxidation and was not itself actively oxidized by monoamine oxidase.

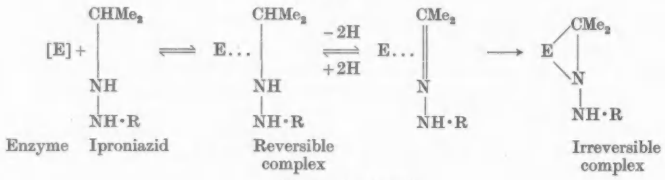
DISCUSSION

In the presence of cyanide mitochondrial monoamine oxidase activity, as measured by oxidation of tyramine, is inhibited by iproniazid at low concentrations (μM). In the absence of cyanide these concentrations of iproniazid have little effect on enzyme activity. Since prior incubation of iproniazid with cyanide in oxygen or with cyanide and mitochondria anaerobically at 37° does not effect the degree of inhibition of the monoamine oxidase activity subsequently obtained, it seems improbable that cyanide reacts in any way with iproniazid. However, it has been shown that inhibition by iproniazid can be greatly increased by shaking the mitochondria with cyanide in oxygen before adding the inhibitor. This sensitization reaches a maximum in 50–60 min., can be readily reversed by washing, requires the presence of oxygen and does not occur in the presence of iproniazid. Inhibition of fully sensitized liver mitochondrial monoamine oxidase by iproniazid is immediate and irreversible.

Since pre-shaking mitochondria with cyanide in oxygen increases the affinity constant of iproniazid for the enzyme, it appears that the site of attachment of the inhibitor on the enzyme is influenced by this treatment. The sensitization can be easily reversed by washing, which suggests that cyanide reacts at the site of attachment and not by the removal of possible interfering factors, such as metal ions.

When iproniazid is added to mitochondrial suspensions containing cyanide in the presence of oxygen, the inhibition which occurs on incubation is greater than in the absence of cyanide. This inhibition is progressive and first-order and cannot be reactivated by washing the mitochondria. However, the results from a dilution experiment suggest that some part of the inhibition may be reversible. In this method of dilution the incubation of iproniazid has to be performed with concentrated mitochondria in the side arm of a Warburg flask and these different conditions may explain the apparent anomaly. Glutathione or the supernatant prepared from rat-liver suspensions when added to mitochondrial suspensions decreases the inhibition of monoamine oxidase obtained by incubation with iproniazid. This effect can be prevented by adding cyanide. These observations suggest that a thiol group may be involved in the attachment of iproniazid to the enzyme. It is therefore of interest that Friedenwald & Herrmann (1942) have shown that monoamine oxidase is a thiol enzyme and that inhibition by mercurials can be reversed by glutathione only in the presence of cyanide.

Higher concentrations of iproniazid (10^{-5} M), in the absence of cyanide, also irreversibly inhibit monoamine oxidase activity both *in vivo* and *in vitro* (Zeller *et al.* 1955*a*). It has been found that for irreversible inhibition to occur *in vitro* pre-incubation of liver mitochondrial monoamine oxidase with iproniazid in oxygen is necessary. The inhibition is first-order, and since it has a high energy of activation it seems likely that a chemical reaction of some kind occurs. Zeller *et al.* (1955*b*) have pointed out that the requisite structure for an inhibitor of monoamine oxidase is $R_2N \cdot NHR'$



(where R is H or other substituent and R′ is an aryl or alkyl group). Since there is evidence to suggest that iproniazid may be metabolized to an inhibitor of a pyridoxal phosphate-requiring enzyme (Davison, 1956), it seemed possible that iproniazid was hydrolysed to *iso*propylhydrazine, and that this substance inhibited both monoamine oxidase and pyridoxal phosphate-requiring enzymes. It was therefore interesting to find that *iso*propylhydrazine is a more powerful inhibitor of monoamine oxidase than iproniazid; however, like iproniazid, *iso*propylhydrazine also requires the presence of oxygen to act as an irreversible inhibitor, and the progressive inhibition reaction has an apparent energy of activation of the same order as for iproniazid. This suggests that the mechanism of the inhibition by iproniazid and *iso*propylhydrazine are similar and that iproniazid is not necessarily converted into *iso*propylhydrazine. This view is confirmed by the finding that the degree of inhibition of monoamine oxidase is not altered by shaking iproniazid in buffer at 37° or with mitochondria in an atmosphere of nitrogen. The susceptibility of monoamine oxidase activity to inhibition by iproniazid is not affected by partial thermal inactivation. Thus if alteration of iproniazid does occur, the system that brings this about must have the same sensitivity to heat as monoamine oxidase. Finally it has been shown that monoamine oxidase inhibited by methyl- or *iso*propyl-hydrazine is not easily reactivated, whereas enzyme inhibited by iproniazid or 1-*iso*nicotinoyl-2-methylhydrazine can be partially reactivated by dialysis or by the addition of dimercaptopropanol.

Another possibility is that iproniazid is converted into *iso*propylhydrazine by monoamine oxidase itself. This would seem unlikely, because Zeller *et al.* (1955*b*) have shown that such substances as 1:2-disubstituted *iso*nicotinoylhydrazines are inhibitors of monoamine oxidase; secondly, *iso*propylhydrazine shows increased inhibition in the presence of cyanide.

An alternative hypothesis for the mechanism of irreversible inhibition of monoamine oxidase activity by iproniazid is that dehydrogenation of iproniazid to 1-*iso*propylidene-2-*iso*nicotinoylhydrazine occurs in the presence of oxygen at the enzyme active centre, in a similar way to that postulated for the first stage in amine oxidation.

Evidence that iproniazid reacts at the active centre is provided by our finding that inhibition is competitive and sensitive to pH change and heat in a similar way to substrate oxidation. Zeller *et al.* (1955*b*) have shown that there must be a free hydrogen atom on the terminal nitrogen bearing the alkyl group of the substituted hydrogen; it therefore seems probable that, once attached to the active centre, the iproniazid undergoes dehydrogenation and reacts further with the enzyme.

SUMMARY

1. Inhibition of rat-liver monoamine oxidase by iproniazid is first-order and irreversible and has a high energy of activation.

2. Since the inhibition reaction has also been found to require oxygen, it is postulated that irreversible inhibition is the result of dehydrogenation of iproniazid at the active centre of the enzyme.

3. In the presence of potassium cyanide inhibition of mitochondrial monoamine oxidase is much increased. This effect is augmented by prior shaking of the mitochondria in oxygen with potassium cyanide.

My thanks are due to Dr A. L. Morrison for his interest in this work, to Mr R. F. Denman for his skilled help and to the Directors of Roche Products Ltd. for permission to publish this work.

REFERENCES

Aldridge, W. N. (1950). *Biochem. J.* **46**, 451.
Aldridge, W. N., Berry, W. K. & Davies, D. R. (1949). *Nature, Lond.*, **164**, 925.
Blaschko, H. (1952). *Pharmacol. Rev.* **4**, 415.
Brodie, B. B., Pletscher, A. & Shore, P. A. (1956). *J. Pharmacol.* **116**, 9.
Davison, A. N. (1956). *Biochim. biophys. Acta*, **19**, 131.
Friedenwald, J. S. & Herrmann, H. (1942). *J. biol. Chem.* **146**, 411.
Furchgott, R. F. (1955). *Pharmacol. Rev.* **7**, 183.
Griesmer, E. C. & Wells, J. A. (1956). *J. Pharmacol.* **116**, 282.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Lochte, H. L., Noyes, W. A. & Bailey, J. R. (1922). *J. Amer. chem. Soc.* **44**, 2556.
Schayer, R. W., Smiley, R. L. & Kaplan, E. H. (1952). *J. biol. Chem.* **198**, 545.
Zeller, E. A., Barsky, J. & Berman, E. R. (1955*a*). *J. biol. Chem.* **214**, 267.
Zeller, E. A., Barsky, J., Fouts, J. R. & Lazanas, J. C. (1955*b*). *Biochem. J.* **60**, v.

Comparative Studies of 'Bile Salts'

10. BILE SALTS OF THE KING PENGUIN, *APTENODITES PATAGONICA**

By I. G. ANDERSON and G. A. D. HASLEWOOD
Guy's Hospital Medical School, London, S.E. 1

and I. D. P. WOOTTON
Postgraduate Medical School, London, W. 12

(*Received* 11 *April* 1957)

The scanty information about the chemical nature of the bile acids of birds has not been previously collected together and this is now done in Table 1. Claims have been made for the detection of free bile acids in bird bile (e.g. Maeda, 1938), but it is believed that these acids generally occur conjugated with taurine. Haslewood & Sjövall (1954) could detect no glycine conjugates in the bile salts of eight species of birds, and Wiggins (1955*a*), in a careful examination of chicken bile by countercurrent distribution methods, failed to find either free bile acids or glycine conjugates.

The present report is of an examination of the bile salts of the King penguin, *Aptenodites patagonica*, and includes remarks on the chemical nature of tetrahydroxynorsterocholanic acid.

RESULTS

By methods previously used in these studies, King penguin bile was shown to contain chenodeoxycholic acid, cholic acid and tetrahydroxynorsterocholanic acid. At least four other 'bile acids' may be present in the bile; none of these appeared to be substances known to us. The proportion of cholic and tetrahydroxynorsterocholanic acids, taken together, was much greater than in chicken bile, but their striking chemical similarity prevented complete separation of these acids or their esters and hence any reliable estimation of the amounts of each.

Ethyl cholate and 'ethyl tetrahydroxynorsterocholanate' ran at virtually the same rate on our paper chromatograms. These esters apparently formed mixed crystals; the melting point of one of our preparations could be raised to about 200° by recrystallization from benzene. Material of this purity on hydrolysis yielded a sample of tetrahydroxynorsterocholanic acid, m.p. about 214°, with characteristic behaviour, apparently substantially the same substance as a sample earlier given to us by Professor T. Kazuno (see Discussion). The ethyl esters of our material and that of Kazuno likewise appeared to be identical. Elementary analyses of our product gave figures corresponding to $C_{26}H_{44}O_5$, i.e. to ethyl cholate, rather than to the

* Part 9: Haslewood (1956).

Table 1. *Bile acids of birds*

Species	Bile acids isolated	Reference
Domestic chicken	Chenodeoxycholic (3α:7α-dihydroxycholanic)	Yonemura (1926); Maeda (1938); Takahashi (1938)
	Isolithocholic	Hosizima, Takata, Uraki & Sibuya (1930)
	Cholic (3α:7α:12α-trihydroxycholanic)	Yamasaki (1933)
	Tetrahydroxynorsterocholanic	Yamasaki (1951)
	3α-Hydroxy-7-oxocholanic	Wiggins (1955*a*)
	3-Oxochola-4:6-dienic*	Wiggins (1955*b*)
Domestic duck (*Anas domestica*); wigeon, *Anas* (*Mareca*) *penelope*; turkey (*Meleagris gallopavo*)	Cholic; chenodeoxycholic	Ishihara & Mori (1938)
Domestic goose	Chenodeoxycholic	Marsson (1849*a*, *b*); Windaus, Bohne & Schwarzkopf (1924)
Pheasant, *Phasianus colchicus karpowi*	Chenodeoxycholic	Ohta (1939*a*)
Owl	Cholic; chenodeoxycholic	Ono & Tamura (1953)
King penguin, *Aptenodites patagonica*	Cholic; chenodeoxycholic; tetrahydroxynorsterocholanic	Present work

* May be an artifact derived from 7α-hydroxy-3-oxochol-4-enic acid (conjugated).

formula $C_{29}H_{50}O_6$ required for the ethyl ester of a 'tetrahydroxynorsterocholanic acid', $C_{27}H_{46}O_6$. The possible significance of these analytical figures is discussed below. An attempt was made to effect further purification of our 'ethyl tetrahydroxynorsterocholanate' by partition chromatography on Celite (kieselguhr) columns, as described by Carpenter & Hess (1956); this was unsuccessful.

Oxidation by chromic acid of a less pure sample of 'ethyl tetrahydroxynorsterocholanate', of m.p. about 180–196°, gave a neutral mixture melting over a wide range. This material was partially separated on Celite columns, giving a pure sample of ethyl dehydrocholate (ethyl 3:7:12-trioxocholanate) and a substance of m.p. 157–166°, together with a mixture. A study of the result of this separation suggested that the ester, m.p. 180–196°, might consist of a binary mixture of about 57% of ethyl cholate and 43% of ethyl tetrahydroxynorsterocholanate: however, the presence of other constituents could not be excluded. Further purification of 'ethyl tetrahydroxynorsterocholanate' was not feasible with the amounts of material available.

Infrared spectra of the chromic oxidation product, m.p. 157–166°, mentioned above and of pure ethyl dehydrocholate were compared. The spectra showed a general similarity, but also well-marked differences. A comparison between the infrared spectra of methyl cholate and of a methylated sample of Kazuno's acid has already been reported (Haslewood & Wootton, 1956).

EXPERIMENTAL

General. Melting points were determined on a Kofler-block type of apparatus, and are corrected. Infrared spectroscopy was carried out as described by Wootton & Wiggins (1953). Al_2O_3 (S) was 'Type H' (Peter Spence and Sons Ltd., Widnes, Lancs.): Al_2O_3 (N) was also from Peter Spence and Sons Ltd., neutralized as described by Shoppee (1949). Light petroleum had b.p. 40–60°, unless otherwise stated. Chromic acid (20%) was a solution of about 20 g. of CrO_3 in a minimal volume of water made, with mixing, to 100 ml. with acetic acid. Microanalyses (C, H) were by Drs Weiler and Strauss, Oxford. Bile salts were isolated as described by Haslewood & Wootton (1950). 'Amyl acetate' was a commercial sample of mixed isomers. It was redistilled and then had b.p. 130–140°.

Paper chromatography of bile salts. With King penguin bile salts, Haslewood & Sjövall (1954) found spots corresponding to taurocholate and taurodeoxycholate in a system not distinguishing between taurodeoxycholate and taurochenodeoxycholate. In the present work, when the method of Sjövall (1955) for separating these substances [descending paper chromatography: mobile phase, 80% (v/v) amyl acetate–heptane; stationary phase, 70% (v/v) acetic acid–water] was used, spots corresponding to taurocholate and taurochenodeoxycholate (not taurodeoxycholate) were detected.

Preparation of crude 'bile acid esters'. King penguin crude bile salts (1 g.) were hydrolysed at 115° for 4 hr. in a sealed metal bomb with 2·5N-NaOH (10 ml.). The acids obtained by acidification of the diluted contents of the bomb after the addition of excess of sodium chloride were washed by manipulating the usually sticky mass under water. Esterification was effected by dissolving the crude acids, freed from excess of water by decantation, in cold ethanol containing 2% (v/v) H_2SO_4 and leaving overnight. The product was isolated by ether extraction of the mixture after dilution with water. The ether was washed with aqueous $NaHCO_3$ and water, dried (Na_2SO_4) and evaporated. Yield of 'crude esters', approx. 560 mg. Acidification of the bicarbonate washings gave no unesterified acid.

Chromatography on alumina

Separation of ethyl esters. 'Crude esters', prepared as above, were dissolved in benzene (about 20 ml.) and the solution was poured onto a column (diam. 14 mm.) of Al_2O_3. Fractions eluted by solvents of varying polarities were examined chromatographically on paper and otherwise as described. Representative results for Al_2O_3 columns of the two types used are given in Tables 2 and 3. Al_2O_3 (S) columns caused some loss of the esters, probably by hydrolysis.

Paper chromatography of column fractions. Methods previously described (Haslewood, 1954) were used. Fractions suspected of containing dihydroxy esters (eluted with ethyl acetate or ether–benzene) were run in system A (Bush, 1952). Later fractions were run in system B_5 or, more successfully, in system B_3 (Bush, 1952). Spot 'X' (Tables 2 and 3) ran at about 68% of the rate of ethyl cholate in system B_3. Combined fractions 31–35 from Al_2O_3 (N) columns showed, in addition to spot 'X', a spot running in system B_3 at a rate slightly slower than that of 'X' and also a spot running at about 25% of the rate of ethyl cholate.

Identification of chenodeoxycholic acid. The gums obtained in fractions 4 and 5 (Table 2) were combined and dissolved in 50% (v/v) acetic acid–acetic anhydride (4 ml.); acetylation was effected by adding, with cooling, 1 drop of 8·5N-perchloric acid. After 15 min. the mixture was diluted with water and extracted with ether. Evaporation of the washed and dried (Na_2SO_4) ether left a gum which was purified by running it through Al_2O_3 (S, 5 g.) in benzene (10 ml.). The benzene-eluted material (226 mg.) crystallized from light petroleum in long colourless needles, m.p. 98–100°. Recrystallization from light petroleum raised the m.p. to 103–104°, not depressed by admixture with authentic ethyl diacetyl chenodeoxycholate (m.p. 103–104°).

Identification of cholic acid. The partly crystalline material from (combined) fractions 6–8 (Table 2) was washed with a little 25% (v/v) ether–light petroleum, and the solid (93 mg.) obtained by filtration was recrystallized from benzene–light petroleum to give needles of m.p. 163°, not depressed by admixture with authentic ethyl cholate. This ester (approx. 10 mg.) in cold acetic acid (2 ml.) was oxidized by adding, with mixing, a few drops of 20% CrO_3 at room temperature. The resultant dehydroester (recovered by dilution with water, extraction with ether and evaporation of the washed and dried extract) was recrystallized from aqueous ethanol to give needles of m.p. 213°, not depressed by authentic ethyl dehydrocholate.

Evidence for the presence of tetrahydroxynorsterocholanic acid. In an early experiment, material eluted from an Al_2O_3 (N) column with 10% (v/v) ethanol–ether was recrystallized from ethyl acetate as fine needles, m.p. 174–175° (Found: C, 71·3; H, 10·3%). This substance ran at the

same speed as ethyl cholate on a paper chromatogram in system B_5 (Bush, 1952) and had a 'fingerprint' (1200–900 cm.$^{-1}$) infrared spectrum almost identical with that of ethyl cholate. Hydrolysis followed by re-esterification with ethanol of this substance gave a product which, after crystallization from benzene–light petroleum, had m.p. 195–198°.

In another experiment, combined fractions [500 mg., 20% (v/v) acetone–ether eluate] from Al_2O_3 (N) columns were left for 2 days with cold ether. The crystalline residue left after decantation of ether was washed with cold ether; it then had m.p. 172–176°. This material was recrystallized from aqueous ethanol, light petroleum–acetone (twice), aqueous ethanol and benzene (in that order), finally giving white needles (14 mg.) of m.p. 196–200° (Found: C, 71·5; H, 10·7. $C_{26}H_{44}O_5$ requires C, 71·6; H, 10·1. $C_{29}H_{50}O_6$ requires C, 70·5; H, 10·1%).

This material (5 mg.) was dissolved by gentle warming and left for 2 hr. in ethanol (0·3 ml.) with 5N-KOH (2 drops) and water (1 drop). The solution was diluted with water and treated with excess of 5N-HCl and of NaCl. The gelatinous precipitate was collected, washed with water and dissolved in ethanol. Evaporation left a residue which was warmed with ethyl acetate (about 1 ml.) and filtered after cooling. Repetition of this process finally removed a persistent amorphous impurity and allowed the preparation of a clear solution in about 0·5 ml. of ethyl acetate. On standing, at about 20° and then 5°, this deposited the bulk of the product as white needles; solvent was decanted and the crystals were washed with cold ethyl acetate. They then had m.p. 198–200°, depressed to 185–194° by a sample of cholic acid (m.p. 196–198°), and showed the same behaviour after melting as Kazuno's tetrahydroxynorsterocholanic acid (see below). In another experiment a similar hydrolysis of a sample of crystalline ester gave after treatment of the product with ethyl acetate white needles of m.p. 211–214°, not depressed by Kazuno's acid (below) and showing the same behaviour after melting as this substance.

Tetrahydroxynorsterocholanic acid, given by Professor T. Kazuno of Okayama Medical School, had m.p. 215–217°; at about this m.p. formation of a small amount of fresh crystals could be seen, and these decomposed at about 298°. Kazuno's acid (1·6 mg.) was left in solution for 2 days with 2% (v/v) H_2SO_4–ethanol (0·2 ml.). The mixture was treated with excess of aqueous $NaHCO_3$ and the solid ester collected and crystallized from dilute ethanol, giving white needles of m.p. 200–203°, not depressed by the above ester, [m.p. 196–200°, prepared from the 20% (v/v) acetone–ether eluates of Al_2O_3 (N) columns]. Both esters ran at the same rate as ethyl cholate on paper in Bush's (1952) system B_3; ethyl 3α:7α:12α-25D-trihydroxycoprostanate ran at more than twice this rate.

Isolation of 'ketonic' fraction. A fraction (240 mg.) corresponding to 4–5 of Table 2 was boiled under reflux for

Table 2. *Alumina chromatography (first type) of ethylated 'bile acids' from King penguin bile*

The crude esters (1050 mg., black gum) were fractionated on 30 g. of Al_2O_3 (S).

Fraction no.	Eluted by (100 ml. for each fraction)	Wt. (mg.)	Appearance	Results of paper chromatography (for methods, see text)
1, 2	Benzene	197	Oil	—
3	Ether	4	Oil	—
4	Ethyl acetate	101	Gum	Spot as ethyl chenodeoxycholate
5	Ethyl acetate	228	Gum	
6–8	Ethyl acetate	157	Partly cryst.	Spot as ethyl cholate
9, 10	Ethyl acetate	50	Solid	—
11	Ethyl acetate	16	Solid	—
12	Ethanol	102	Solid	Spot as ethyl cholate and spot 'X' (see text)
13	Ethanol	2	Solid	—
Total eluted (see text)		857	—	—

Table 3. *Alumina chromatography (second type) of ethylated 'bile acids' from King penguin bile*

The crude esters (731 mg.) were fractionated on 7·3 g. of Al_2O_3 (N) (see text).

Fraction no.	Eluted by (solvent, ml. total vol.)	Wt. (mg.)	Appearance	Results of paper chromatography (for methods, see text)
1	Benzene, 100	316	Oil	Spot as ethyl chenodeoxycholate
2–8	Benzene, 350	58	Oil	
9, 10	10% (v/v) ether–benzene, 150	19	Gum	
11, 12	20% (v/v) ether–benzene, 150	12	Gum	Spots as ethyl chenodeoxycholate and ethyl cholate
13, 14	50% (v/v) ether–benzene, 150	12	Gum	
15	Ether, 100	1	Gum	
16, 17	20% (v/v) acetone–ether, 200	152	Partly cryst.	Spot as ethyl cholate and (?) unknown substance
18–26	20% (v/v) acetone–ether, 450	48	Gum	
27–30	50% (v/v) acetone–ether, 200	41	Gum	Spot as ethyl cholate and 'X' (see text)
31, 32	Acetone, 100	28	Gum	
33, 34	10% (v/v) ethanol–acetone, 150	31	Gum	Spots as ethyl cholate, 'X' and at least 2 other compounds (see text)
35	25% (v/v) ethanol–acetone, 50	8	Gum	
36	Ethanol, 50	Trace	—	—
Total eluted		726	—	—

0·75 hr. with Girard's reagent T (100 mg.) in ethanol (3·0 ml.) with acetic acid (0·3 ml.). The oily 'ketones', isolated in the usual way, weighed 65 mg. This material in ethanol (5 ml.) was warmed with 5N-KOH (0·5 ml.) for 0·5 hr. Dilution with water and acidification with 2N-HCl gave semisolid acids which were collected, washed and dissolved in a minimal amount of ethyl acetate. After 9 days solvent was decanted from crystals: the latter (about 1 mg.) had m.p. 186–192°, and from aqueous ethanol gave colourless crystals of m.p. 194–199°. These were methylated with ethereal diazomethane, and the residue, after removal of solvent, was dissolved in CS_2. In the range 1200–900 cm.$^{-1}$ the infrared spectrum resembled closely that of methyl deoxycholate (methyl 3α:12α-dihydroxycholanate) and in the region 1800–1650 cm.$^{-1}$ only one band was present, which was attributed to the carboxylic acid ester. The nature of this substance, evidently carried over from the 'non-ketones', has not been further investigated.

Partition chromatography

An attempt was made to separate ethyl cholate and ethyl tetrahydroxynorsterocholanate and their chromic oxidation products on Celite columns as follows. Celite (kieselguhr, Hyflo Supercel, Johns-Manville Co. Ltd.) was washed with acid and water as described by Carpenter & Hess (1956) and then dried at 60°. For each mixture solvent systems were first found in which the partition coefficient moving phase/stationary phase was about 0·2. The stationary phase was mixed with the Celite and the whole transferred with excess of moving phase: the column was packed as described by Howard & Martin (1950). Substances to be separated were dissolved in not more than 0·7 ml. of stationary phase; the solution was run on 0·1 g. of dry Celite, lightly packed on the top of the column as soon as supernatant moving phase had drained from it. Carpenter & Hess's (1956) theoretical treatment enabled a prediction to be made of the volume (V_E) needed to elute the desired compounds, and fractions of 2 ml., embodying V_E, were collected automatically into weighed tubes. Table 4 gives details of columns; Carpenter & Hess's terms are used: V_S = vol. of stationary phase; $V_{sup.}$ = wt. (g.) of Celite/density of Celite (1·8); $\pi r^2 h$ = vol. of column. The results are shown in Table 5.

Preparation and chromatography of oxidation product (Celite columns 4 and 5 of Tables 4 and 5). The mixture of ethyl esters (11 mg., m.p. 178–200°) recovered from Celite column no. 3 (Table 5) in acetic acid (0·4 ml.) was left with

Table 4. *Details of partition columns on Celite*

For explanation of symbols see text. The parts by volume of solvents given were mixed and equilibrated in a separating funnel. The lower phase was then used as the stationary, and the upper as the moving, phase.

Celite column no.	System (parts by vol.)	Substance put on column (mg. in ml. of stationary phase)	Wt. of Celite (g.)	V_S (ml.)	$\pi r^2 \times h$ (cm.^{2} × cm.)	Rate of flow (ml./hr.)
1	Heptane, 5: benzene, 5; ethanol, 10; water, 4	Ethyl cholate (21 in 0·3)	9	5	1·29 × 16·4	5·2
2	As col. no. 1	King penguin ethyl esters, crystallized from benzene to m.p. about 180–196° (17 in 0·3)	9	5	1·29 × 16·4	5·2
3	As col. no. 1	As above (17 in 0·3)	36	20	1·4 × 70	11·0
4	Light petroleum (b.p. 83–95°), 4; benzene, 1; ethanol, 5; water, 1	Chromic oxidation product of the above (described in text; 10 in 0·7)	9	5	1·4 × 17·9	15·0
5	As col. no. 4	As above (11 in 0·7)	36	20	1·4 × 73	11·0

Table 5. *Results of partition chromatography on Celite columns described in Table 4*

Fractions of 2 ml. of effluent were collected.

Celite column no.	Vol. (ml.) of effluent (V_E) giving crystals		Eluted crystals: Wt. (mg.)	Eluted crystals: Approx. m.p.	Character of graph: mg. eluted/ml. V_E/V_E	Remarks
1	24–34		21	163–164°	Single sharp peak	Probably the m.p. of pure ethyl cholate
2	22–34		17	178–200°	Single sharp peak	At peak, 2 ml. fraction contained about 4 mg.
3	115–143		17	178–200°	Single sharp peak	At peak, 2 ml. fractions contained about 2 mg.
4	42–50		2·7	214–227°	Sharp peak with later tailing	For possible interpretation, see text
	52–60		3·8	140–216°		
	62–66		1·6	148–158°		
5	176–208	(*A*)	7·3	180–226°	Broad peak with considerable tailing	No fraction contained more than about 0·5 mg. For further treatment of *A* and *B*, see text
	210–250	(*B*)	3·0	156–166°		

20% CrO_3 (0·1 ml.) for 15 min. Excess of water and then NaCl were added, and the precipitate was extracted with ether. The ether was washed with water, aqueous NH_3 and water, and dried (Na_2SO_4) and evaporated. The residue (9·8 mg.) had m.p. about 135–195°. Chromatography of this material gave results shown in Table 5; these are further discussed below.

For the Celite column no. 4 (Table 5), if the 2·7 mg. portion, m.p. 214–227°, is taken as ethyl dehydrocholate, the 1·6 mg. portion, m.p. 148–158°, as a substance which when pure would melt at (say) about 170°, and the 3·8 mg., m.p. 140–216°, as being composed of equal weights of the two compounds, then the approximate composition of the mixture recovered from the column was: ethyl dehydrocholate (2·7 + 3·8/2 = 4·6 mg.), 57%; substance of m.p. about 170° (1·6 + 3·8/2 = 3·5 mg.), 43%.

Eluted material was further treated as follows: Fractions *A* (Celite column no. 5, Table 5) were combined and recrystallized from aqueous ethanol, giving long colourless needles (4 mg.), m.p. 221–223°, not depressed by ethyl dehydrocholate (m.p. 221–223°); fractions *B*, from the same column, similarly treated, gave fine colourless needles (2 mg.)., m.p. 157–166°; these (1 mg.) were dissolved in CS_2 and the infrared spectrum of the solution in the range 1200–900 $cm.^{-1}$ was compared with that similarly obtained from the above specimen of ethyl dehydrocholate. There were definite differences in the spectra, in spite of an overall similarity.

DISCUSSION

Biological. The evolutionary position of penguins has been fully discussed by Simpson (1946), and the consensus of opinion appears to be that there are no adequate grounds for believing that the line of descent of these birds from reptiles differs from that of the other Carinate birds. Our findings do not conflict with this view, for there is clearly a close resemblance between the bile salts of the King penguin and those of the other birds mentioned in Table 1. The only obviously different feature is the occurrence in King penguin bile of tetrahydroxynorsterocholanic acid and probably of cholic acid in amounts considerably greater than have been found in the bile of other birds. In preliminary work we have found the same to be true for the bile salts of the Gentoo penguin, *Pygoscelis papua*. This difference may have a dietary origin; however, we think it desirable to defer further discussion of the biological significance of the differences in bird bile acids until many more species have been examined and the chemical nature of tetrahydroxynorsterocholanic acid has been elucidated.

Chemical. The most striking chemical finding in the present work is the great similarity between cholic and tetrahydroxynorsterocholanic acids and their ethyl esters. Ohta's (1939*b*) tetrahydroxynorsterocholanic acid was isolated from the bile of a fish, and so presumably was that used by Isaka (1940). The sample sent to us by Professor Kazuno was, we believe, obtained from chicken bile by Yamasaki (1951), who put forward the view that it is identical with Ohta's acid. Our new material is undoubtedly substantially the same as, though less pure than, Kazuno's sample. Our analyses suggest that ethyl tetrahydroxynorsterocholanate may have the formula $C_{26}H_{44}O_5$: however, the analytical sample certainly contained some ethyl cholate. If the formula $C_{26}H_{44}O_5$ proves to be correct, the parent acid, $C_{24}H_{40}O_5$, is an isomer of cholic acid. Such isomerism could lie at an asymmetric centre or centres, for example, at C-17 or C-20 in the steroid nucleus. The isomerism, if it exists, is not concerned only with the nuclear hydroxyl groups (which we believe on spectroscopic evidence to be at 3α:7α and 12α: Haslewood & Wootton, 1956), for if it resided in these groups alone the product obtained by oxidation of 'ethyl tetrahydroxynorsterocholanate' with chromic acid would have been ethyl dehydrocholate, instead of containing, as it did, a large proportion of a quite different substance. We defer further discussion on the chemical nature of tetrahydroxynorsterocholanic acid until more material is available. It is clear that King penguin bile also contains a number of hitherto unknown substances, which may be bile acids. Examples of these are: the substance giving spot 'X' (Table 2), the two compounds mentioned in fractions 33–35 of Table 3 and the non-ketonic substance finally giving an infrared spectrum like that of methyl deoxycholate, described on p. 326.

SUMMARY

1. The bile salts of the King penguin (*Aptenodites patagonica*) have been shown to contain chenodeoxycholic, cholic, tetrahydroxynorsterocholanic and at least four other (unidentified) acids. The proportion of 'cholic–tetrahydroxynorsterocholanic' acids was greater than that reported in the bile of other birds. Apart from this, King penguin bile salts resembled those of the other birds examined by previous workers.

2. An unsuccessful attempt has been made to separate completely by crystallization and partition chromatography the constituents of the 'ethyl cholate–ethyl tetrahydroxynorsterocholanate' mixture from King penguin bile salts. The purest sample of 'ethyl tetrahydroxynorsterocholanate' prepared by us from this mixture had m.p. 196–200°, gave analytical figures in agreement with $C_{26}H_{44}O_5$, and was hydrolysed to a mixture consisting largely of 'tetrahydroxynorsterocholanic acid', apparently identical with a sample earlier given to us by Professor T. Kazuno. Ethyl cholate, $C_{26}H_{44}O_5$, similarly recovered from partition chromatography on Celite (kieselguhr) columns, had m.p. 163–164°.

3. Chromic oxidation of impure 'ethyl tetrahydroxynorsterocholanate', m.p. 180–196°, gave a mixture from which was obtained a specimen of

pure ethyl dehydrocholate, m.p. 221–223°, and an impure but different ester, m.p. 157–166°. The infra-red spectra of these two substances in the range 1200–900 cm.$^{-1}$ showed an overall similarity but also definite differences.

The authors offer their thanks to the following, without whose generous help in collecting King penguin bile this work could not have been undertaken: Dr E. C. Appelby and the Royal Zoological Society of Scotland, and Bernard Stonehouse of the Falkland Islands Dependencies Survey and the Edward Grey Institute of Field Ornithology, Oxford.

REFERENCES

Bush, I. E. (1952). *Biochem. J.* **50**, 370.
Carpenter, F. H. & Hess, G. P. (1956). *J. Amer. chem. Soc.* **78**, 3351.
Haslewood, G. A. D. (1954). *Biochem. J.* **56**, 581.
Haslewood, G. A. D. (1956). *Biochem. J.* **62**, 637.
Haslewood, G. A. D. & Sjövall, J. (1954). *Biochem. J.* **57**, 126.
Haslewood, G. A. D. & Wootton, I. D. P. (1956). *Biochem. J.* **63**, 3P.
Haslewood, G. A. D. & Wootton, V. (1950). *Biochem. J.* **47**, 584.
Hosizima, T., Takata, H., Uraki, Z. & Sibuya, S. (1930). *J. Biochem., Tokyo*, **12**, 393.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Isaka, H. (1940). *Hoppe-Seyl. Z.* **266**, 117.
Ishihara, T. & Mori, T. (1938). *Arb. med. Univ. Okayama*, **5**, 538.
Maeda, K. (1938). *Arb. med. Univ. Okayama*, **6**, 101.
Marsson, Th. (1849*a*). *Liebigs Ann.* **72**, 317.
Marsson, Th. (1849*b*). *Arch. Pharm., Berl.*, **58**, 138.
Ohta, K. (1939*a*). *Arb. med. Univ. Okayama*, **6**, 193.
Ohta, K. (1939*b*). *Hoppe-Seyl. Z.* **259**, 53.
Ono, K. & Tamura, R. (1953). *J. Jap. biochem. Soc.* **25**, 112.
Shoppee, C. W. (1949). *J. chem. Soc.* p. 1671.
Simpson, G. G. (1946). *Bull. Amer. Mus. nat. Hist.* **87**, 1.
Sjövall, J. (1955). *Ark. Kemi*, **8**, 299.
Takahashi, K. (1938). *Hoppe-Seyl. Z.* **255**, 277.
Wiggins, H. S. (1955*a*). Ph.D. Thesis: University of London.
Wiggins, H. S. (1955*b*). *Biochem. J.* **60**, ix.
Windaus, A., Bohne, A. & Schwarzkopf, E. (1924). *Hoppe-Seyl. Z.* **140**, 167.
Wootton, I. D. P. & Wiggins, H. S. (1953). *Biochem. J.* **55**, 292.
Yamasaki, K. (1933). *J. Biochem., Tokyo*, **18**, 323.
Yamasaki, K. (1951). *J. Biochem., Tokyo*, **38**, 93.
Yonemura, S. (1926). *J. Biochem., Tokyo*, **6**, 287; **8**, 79.

The Effect of Sheep-Rumen Contents on Unsaturated Fatty Acids

By F. B. SHORLAND, R. O. WEENINK,
Fats Research Laboratory, Wellington

A. T. JOHNS
Plant Chemistry Laboratory, Palmerston North

and I. R. C. McDONALD
Dominion Laboratory, Department of Scientific and Industrial Research, Wellington, New Zealand

(*Received* 19 *March* 1957)

Diets rich in unsaturated oils modify the depot fats of most animals to resemble the dietary fats, but ruminant depot fats are relatively unaffected (cf. Shorland, 1955). In addition, ruminant fats contain higher proportions of fully saturated glycerides than would be expected on the basis of the even or widest distribution rule of Hilditch (1956). Other peculiarities of ruminant fats include the presence of substantial amounts (5–10%) of *trans* unsaturated acids (Hartman, Shorland & McDonald, 1954, 1955) and of mono- and di-unsaturated acids which differ from oleic and linoleic acids in the position of their double bonds (cf. Shorland, 1956). Hilditch (1956) has suggested that the peculiarities of the glyceride structure of ruminant fats may be due to the hydrogenation *in situ* of preformed oleoglycerides. However, while this process may possibly occur, the most important factor is probably the hydrogenation of the dietary unsaturated fatty acids by the rumen micro-organisms, giving rise to less-unsaturated acids and stearic acid.

Perhaps the first observation of the effect of the rumen on the dietary fat was made by Reiser (1951). He reported that linolenic acid present in linseed oil was converted *in vitro* into linoleic acid by the action of sheep-rumen contents. Similar results were obtained by Hoflund, Holmberg & Sellman (1956), using fistulated sheep.

As a result of the observation of Willey, Riggs, Colby, Butler & Reiser (1952) that the depot fats of steers feed on cottonseed oil contained more stearic acid than the controls, R. Reiser (private communication) suggested that the high content of stearic acid in the depot fats of ruminants was due to the hydrogenation of dietary C_{18} unsaturated acids in the rumen. This suggestion was further supported

by Shorland, Weenink & Johns (1955), who showed that whereas linolenic acid was the main component of pasture lipids, in pasture-fed sheep the rumen contents contained stearic acid as the main lipid constituent. Furthermore, incubation of linolenic acid with sheep-rumen contents produced mainly stearic acid and not linoleic acid, as originally reported by Reiser (1951). Recently, Reiser & Reddy (1956) fed cottonseed oil and linseed oil to goats and found that the linoleic and linolenic acids present were decreased to a very low level in the rumen and increased amounts of saturated fatty acids were formed.

To provide more precise information about the hydrogenating action of the rumen micro-organisms, we now report the effect of sheep-rumen contents on oleic, linoleic and linolenic acids.

EXPERIMENTAL

Fatty acids used for incubation experiments

Oleic acid. The oleic acid, iodine value 90·1, was prepared from olive oil as described by Brown & Shinowara (1937).

Linoleic acid. This acid was prepared from cottonseed oil as described by Hilditch, Patel & Riley (1951). The iodine value was 173·7 (theoretical, 181·4). Analysis by the Brice, Swain, Herb, Nichols & Riemenschneider (1952) procedure showed the presence of 92·9% of non-conjugated dienoic acids, together with 0·2% of conjugated dienoic acids. Trienoic acids were not detected.

Linolenic acid. This was prepared from conophor oil, by the urea-complex procedure of Swern & Parker (1953). The linolenic acid concentrate was purified by fractional distillation of the methyl esters followed by adsorption chromatography as described by Reinbold & Dutton (1948). The acids finally obtained had an iodine value of 264·8 as compared with 274·1 required for pure linolenic acid. Analysis by the procedure of Brice *et al.* (1952) showed the presence of 91·3% of non-conjugated trienoic, 3·9% of non-conjugated dienoic, 1·9% of conjugated trienoic and 1·4% of conjugated dienoic acids.

By using the technique of Hartman *et al.* (1955) it was found that only negligible amounts of *trans* unsaturated compounds (less than 1% calculated as elaidic acid) were present in all of the above acids.

Incubation of the fatty acids

Fistulated sheep maintained on a ryegrass–white clover pasture were used as a source of rumen contents. Approximately 1 l. of contents was removed from each sheep after the animals had been withheld from pasture for 6 hr. to produce reasonably fluid contents. The samples were mixed under anaerobic conditions. The rumen contents were not strained, as it was considered that the fermentation of the residual plant material would be necessary for the production of hydrogen for the hydrogenation reaction. The combined rumen samples were divided into portions of 700 ml. Of these, one was used for the control experiment, and to the remainder 5 g. samples of oleic, linoleic and linolenic acids were added. They were incubated in 1 l. Erlenmeyer flasks, fitted with rubber bungs through which passed tubing for gassing and sampling, as well as a valve to allow the escape of fermentation gases. The flasks were gassed with carbon dioxide and incubated at 37° for 2 days, being shaken at hourly intervals by hand during the day. Samples were withdrawn via the sampling tube at approximately 3 hr. intervals on the first day and once on the second day for determination of pH.

The pH values of the contents of the flask were kept within the range 6·5–5·8 by the addition, when necessary, of solid sodium bicarbonate to the flask. This was done by quickly removing the rubber bung, adding the bicarbonate, replacing the bung, shaking and regassing with carbon dioxide. The pH range was chosen as that found in normal rumen contents of pasture-fed sheep at the Plant Chemistry Laboratory.

The reactions were stopped by adding an equal volume of ethanol and heating to boiling point. The aqueous ethanol was evaporated *in vacuo* on the water bath and the residue extracted first with acetone and then continuously with ethyl ether. The extracts were evaporated, dissolved in ethyl ether and washed with water, and the total lipids recovered by evaporation of the ether on the water bath. The last traces of solvent and water were removed *in vacuo* at 100°.

Analysis of lipid fractions

The total lipid fractions of the control and experimental samples were saponified and the unsaponifiable matter was extracted as outlined in the standard method of the Society of Public Analysts (1933). The aqueous solutions were acidified and the fatty acids recovered by extraction with ethyl ether. Under these conditions of saponification the amounts of conjugated acids formed from linolenic acid are less than 1%. The methyl esters of the fatty acids, prepared as described by Hilditch (1956), were separated into 'solids' and 'liquids' by crystallization from 15 vol. of acetone three times at −35° (Shorland & De la Mare, 1945). The methyl esters of the solids and liquids were fractionated by using column E, described by Shorland (1952), and the proportions of C_{18} constituents determined from the saponification equivalents and iodine values of the fractions. The proportions of diene and triene constituents were determined by the technique of Brice *et al.* (1952). By means of the infrared method described by Hartman *et al.* (1955) the contents of *trans* double bond expressed as elaidic acid were measured from the graphs of 5% (w/v) solutions of the methyl esters in carbon disulphide, compensated with a similar concentration of methyl palmitate in the same solvent. Fractions particularly rich in methyl esters of *trans* acids (greater than 30%) were measured as above but at a lower concentration, namely 1% (w/v).

The results of the incubation experiments shown in Table 1 have been expressed in terms of the differences between the weights of the various C_{18} fatty acid constituents present in the control sample and in the samples to which the fatty acids have been added. The amount of added C_{18} unsaturated acids was about four times that present in the control experiment.

In view of the substantial amounts of dienoic acids, both conjugated and non-conjugated (see Table 1), after the incubation of linoleic acid the infrared absorption spectra were re-examined for

Table 1. *The component fatty acids formed during incubation of unsaturated fatty acids with sheep-rumen contents*

The figures in parentheses denote the amounts formed, expressed as a percentage of the wt. of the added acid. The composition of the fatty acids in the control sample (no acid added) was as follows (wt. %): saturated C_{16}, 14·1; C_{18}, 50·5; C_{20} and above, 3·2. Unsaturated C_{14}, 1·4; C_{16}, 1·6; C_{18} monoene, 28·4; C_{18} non-conjugated diene 0·1, triene 0·4; C_{18} conjugated diene 0·3, triene trace.

Fatty acid added		Fatty acids formed (g.)						
			C_{18} unsaturated					
				Non-conjugated†		Conjugated		
Nature	Wt. (g.)*	Stearic	Monoene	Diene	Triene	Diene	Triene	*trans* (as elaidic)
Oleic	5·00	1·13 (22·6)	2·95 (59·0)	0·03 (0·6)	0·05 (1·0)	0·07 (1·4)	0·04 (0·8)	0·86 (17·2)
Linoleic	20·00	3·13 (15·6)	8·94 (44·7)	3·77 (18·9)	0·03 (0·2)	3·00 (15·0)	0·05 (0·3)	9·58 (47·9)
Linolenic	20·00	3·37 (16·8)	14·45 (72·2)	Trace —	0·14 (0·7)	0·29 (1·4)	Trace Trace	13·47 (67·3)

* Uncorrected for impurities.
† As customarily designated but preferably termed conjugatable under experimental conditions used.

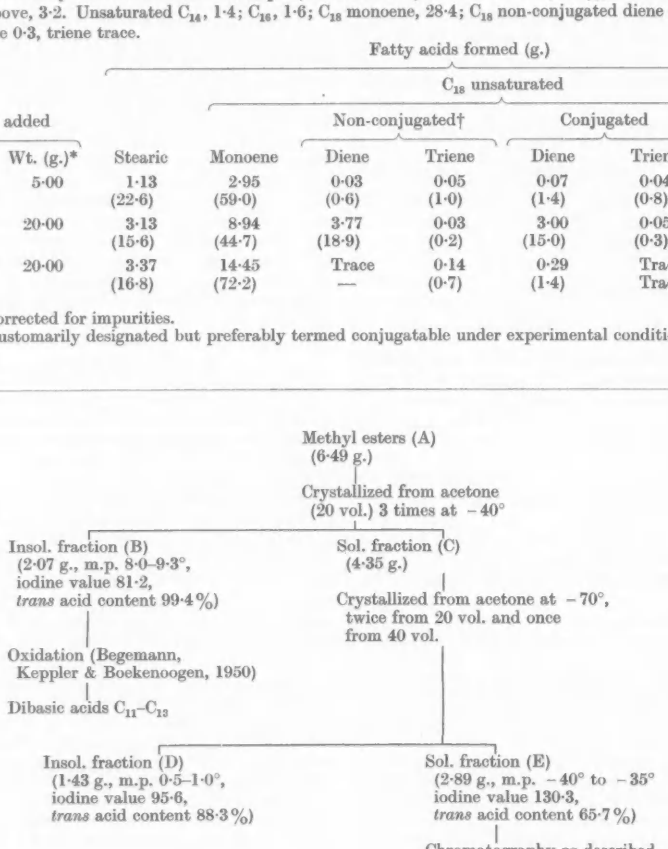


Fig. 1. Separation of a C_{18} non-conjugatable dienoic acid concentrate after the incubation of linolenic acid with sheep-rumen contents.

the presence of conjugated *trans* acids. From the extinction-coefficient and peak-frequency data of Ahlers, Brett & McTaggart (1953) it was calculated that 5·3 % of *trans–trans* conjugated and 2·0 % of *cis–trans* conjugated dienoic acids were present.

Carotene was determined according to method B of the Grass Driers' Association (1941).

The non-conjugatable dienoic acids from the linolenic acid incubation experiment

The Wijs iodine values and the calculated iodine values (Brice *et al.* 1952) of the C_{18} unsaturated fraction from the oleic and linoleic experiments agreed, but there was a wide discrepancy in the linolenic acid experiment. Here the Wijs iodine value was 107·4 and the calculated value 89·5. This difference, if calculated as dienoic acid, represents about 21 % of the fraction.

The methyl esters of C_{18} unsaturated acids from the linolenic acid experiment (denoted A) were therefore separated as shown in Fig. 1 for further examination.

From the results given in Fig. 1 the main concentrate of mono-unsaturated acids appears to consist of a mixture of *trans*-Δ^{11}-, Δ^{12}- and Δ^{13}-octadecenoic acids. Fraction E 96 (0·61 g.) appeared from the iodine value to contain considerable proportions of dienoic acid, and was therefore selected for further investigation. Analyses by the Brice *et al.* (1952) procedure showed 2·8 % of conjugatable trienoic acids together with traces of conjugated dienoic and trienoic acids. However, assuming the presence of mono- and di-enoic constituents only, it may be calculated from the iodine value of 157·1 that the content of methyl octadecadienoate was 82 %.

Ozonolysis of methyl linoleate (m.p. of tetrabromide of acids 114°) by the procedure of Klenk & Bongard (1952) followed by chromatography of dibasic acids as described by Marvel & Rands (1950) gave malonic acid only. Under similar conditions E 96 gave the following molar percentages of dibasic acids, based on the weight of ester taken: malonic, 3·6; succinic, 17·0; glutaric, 2·0; adipic, 1·2. These results show that the main dienoic constituent present in fraction E 96 contains two methylenic groups between the double bonds. The presence of some malonic acid is to be expected from the residual conjugatable trienoic acid.

In determining the monobasic acids the oxidation procedure of Allen & Kiess (1955) was used. The resulting acids were determined by the method of James & Martin (1952). A linolenic acid concentrate containing 69·8 % of linolenic acid, 18·8 % of linoleic acid and 11·4 % of oleic acid gave the following molar percentages of monobasic acids: propionic acid, 70·0; hexanoic acid, 19·5; nonanoic acid and higher fatty acids, 10·5. In a sample of fraction E 96 there were found the following molar percentages: propionic acid, 64; butyric acid, 20; hexanoic acid, 16.

DISCUSSION

The object of the fermentation *in vitro* was to examine qualitatively the conversion of unsaturated acids by rumen organisms without the necessity of preparing the larger amounts of materials that are necessary to dose directly into the animal. The difficulty with a fermentation *in vitro* technique in this case is that a relatively large amount of the acids is necessary for the methods of analysis employed. The most satisfactory method of incubating rumen contents *in vitro* appears to be the use of a semipermeable sac in which rumen liquor is dialysed against a mineral solution (Warner, 1956). However, it was thought that the number and size of the 'artificial rumens' necessary to carry out the experiment with the amount of substrate involved made their use impracticable. Hence recourse was made to the all-glass impermeable system. As the pasture on which the sheep were feeding contains the acids as substrates in the present experiments, the bacteria necessary for their hydrogenation should be present in the rumen ingesta.

As shown in Table 1, 15·6–22·6 % of stearic acid was formed when oleic, linoleic or linolenic acids were incubated with rumen contents. The change in composition of the added fatty acid was least marked with oleic acid, 59·0 % remaining unhydrogenated as compared with 18·9 % for linoleic acid and 0·7 % for linolenic acid. These changes were paralleled by *trans* acid formation, which amounted to 17·2, 47·9 and 67·3 % from oleic acid, linoleic acid and linolenic acid respectively (see Table 1). In the linoleic acid experiment, of the total (33·9 %) dienoic acid remaining, 15·0 %, or nearly half, had been converted into the conjugated form. If these results are compared with those reported by Reiser & Reddy (1956) for steers, it appears that in our experiments the degree of hydrogenation of linoleic acid was similar, but that linolenic acid was more completely hydrogenated, with not more than traces of di- and tri-enoic acids remaining as determined by alkali-isomerization technique. However, hydrogenation of linolenic acid led to the formation of non-conjugatable dienoic acids.

The formation of *trans* and positional isomers of unsaturated fatty acids is known to occur during the industrial hydrogenation of natural fats (cf. Markley, 1947). The mechanism of the reactions involved is not completely understood, but in the simplest case, the hydrogenation of methyl oleate, Allen & Kiess (1955) have shown that the double bond migrates equally in either direction along the

carbon chain with the formation of positional and geometrical isomers. In the hydrogenation of methyl linoleate Allen & Kiess (1956) consider that the methylene-interrupted system shifts to a considerable extent to a conjugated system before hydrogenation takes place. On the other hand, there is evidence that in the hydrogenation of linolenic acid the central double bond is attacked preferentially, leading to the formation of dienoic acids with the double bond separated by more than one methylenic group (cf. Rebello & Daubert, 1951). It could be expected therefore that, if the hydrogenation by the rumen micro-organisms parallels catalytic hydrogenation, the dietary unsaturated acids consisting mainly of linolenic acid could be expected to produce a wide variety of dienoic and monoenoic acids in addition to the oleic and linoleic acids normally found in natural fats.

This expectation appears to be realized; *trans* acids were formed in all cases, conjugated dienoic acids resistant to hydrogenation were present only in the linoleic acid experiment and non-conjugatable dienoic acids were produced by the incubation of linolenic acid (see Table 1). In the latter experiment several isomers with isolated double bonds were present. As propionic and succinic acids were the main products isolated after ozonolysis, octadeca-11:15-dienoic acid would appear to predominate. The presence of octadecadienoic acids with separated double bonds has been inferred by other workers (Garton, 1954), and such acids have been isolated from lamb caul fat (Weenink, 1956). Though analogy between catalytic hydrogenation and hydrogenation by micro-organisms has been demonstrated in this work, there are differences, suggesting that the hydrogenases present in the rumen micro-organisms are to some extent specific. The more complete hydrogenation with accompanying isomerization of linolenic acid as compared with linoleic acid indicates that the octadecadienoic acids (mainly non-conjugatable) formed by the hydrogenation of linolenic acid are more readily hydrogenated under the conditions prevailing in the rumen than is linoleic acid itself. No evidence has been found for the formation of linoleic acid from linolenic acid by rumen micro-organisms.

A further example of the apparent resistance of conjugated systems to hydrogenation by rumen micro-organisms is indicated by the fact that considerable proportions of carotene remain in the rumen contents after prolonged incubation. Our earlier unpublished results showed that the carotene content of ingested pasture is 8·0 mg./g. of unsaponifiable matter as compared with 2·1 mg./g. of unsaponifiable matter in the sheep-rumen contents. In the present work, in each experiment, as well as in the control, the carotene content remained constant at about 1·8 mg./g. of unsaponifiable matter.

The results in Table 1 support the conclusion that the di- and tri-unsaturated acids of the diet are considerably hydrogenated, so that the smallness of the proportions (2 % or less of the fat) of di- and poly-enoic acids in the depot fats of ruminants can be explained.

The amount of *trans* double bonds in the linolenic acid experiment when based on the unsaturated acid content was approximately 91 %. This is higher than was found in the equilibrium mixture (66 %) when methyl oleate was catalytically hydrogenated (Feuge, Pepper, O'Connor & Field, 1951) but comparable with that obtained (89 %) for methyl linoleate (Feuge, Cousins, Fore, Dupre & O'Connor, 1953) in similar circumstances.

Shorland *et al.* (1955) found that in the rumen of fistulated sheep 50 % of the pasture linolenic acid was converted into stearic acid. In the present work we have found that only 16·8 % of the linolenic acid was converted into stearic acid. It appears therefore that *in vivo* the hydrogenation is more complete. However, the present results give an indication of the differences in the facility with which different unsaturated fatty acids are hydrogenated and add to our knowledge of the many different types of unsaturated acids occurring in ruminant fats but not found in non-ruminant fats or vegetable oils.

SUMMARY

The incubation of oleic, linoleic or linolenic acid with rumen contents of sheep in a carbon dioxide atmosphere at 37° resulted in the production of stearic acid as well as *trans* and positional isomers of the unsaturated acids. These results further support the view that many of the peculiarities of ruminant fats arise from the hydrogenation of the dietary unsaturated acids, particularly linolenic acid.

The authors are indebted to Mr C. N. Hooker of the Fats Research Laboratory for the estimation of diene and triene acids as well as for the determination of carotene.

REFERENCES

Ahlers, N. H. E., Brett, R. A. & McTaggart, N. G. (1953). *J. appl. Chem.* **3**, 433.

Allen, R. R. & Kiess, A. A. (1955). *J. Amer. Oil Chem. Soc.* **32**, 400.

Allen, R. R. & Kiess, A. A. (1956). *J. Amer. Oil Chem. Soc.* **33**, 419.

Begemann, P. H., Keppler, J. G. & Boekenoogen, H. A. (1950). *Rec. Trav. chim. Pays-Bas*, **69**, 439.

Brice, B. A., Swain, M. L., Herb, S. F., Nichols, P. L. jun. & Riemenschneider, R. A. (1952). *J. Amer. Oil Chem. Soc.* **29**, 279.

Brown, J. B. & Shinowara, G. Y. (1937). *J. Amer. chem. Soc.* **59**, 6.

Feuge, R. O., Cousins, E. R., Fore, S. P., Dupre, E. F. & O'Connor, R. T. (1953). *J. Amer. Oil Chem. Soc.* **30**, 454.
Feuge, R. O., Pepper, M. B. jun., O'Connor, R. T. & Field, E. T. (1951). *J. Amer. Oil Chem. Soc.* **28**, 420.
Garton, G. A. (1954). *J. Sci. Fd Agric.* **5**, 251.
Grass Driers' Association (1941). *Analyst*, **66**, 334.
Hartman, L., Shorland, F. B. & McDonald, I. R. C. (1954). *Nature, Lond.*, **174**, 185.
Hartman, L., Shorland, F. B. & McDonald, I. R. C. (1955). *Biochem. J.* **61**, 603.
Hilditch, T. P. (1956). *The Chemical Constitution of Natural Fats*, 3rd ed. London: Chapman and Hall, Ltd.
Hilditch, T. P., Patel, C. B. & Riley, J. P. (1951). *Analyst*, **76**, 81.
Hoflund, S., Holmberg, J. & Sellman, G. (1956). *Cornell Vet.* **46**, 53.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
Klenk, E. & Bongard, W. (1952). *Hoppe-Seyl. Z.* **290**, 181.
Lemon, H. W. (1949). *Canad. J. Res.* B, **27**, 605.
Markley, K. S. (1947). *Fatty Acids*, 1st ed. New York & London: Interscience Publishers.
Marvel, C. S. & Rands, R. D. jun. (1950). *J. Amer. chem. Soc.* **72**, 2642.
Rebello, D. & Daubert, B. F. (1951). *J. Amer. Oil Chem. Soc.* **28**, 177.
Reinbold, C. L. & Dutton, H. J. (1948). *J. Amer. Oil Chem. Soc.* **25**, 120.
Reiser, R. (1951). *Fed. Proc.* **10**, 236.
Reiser, R. & Reddy, H. G. R. (1956). *J. Amer. Oil Chem. Soc.* **33**, 155.
Shorland, F. B. (1952). *J. appl. Chem.* **2**, 438.
Shorland, F. B. (1955). *Progress in the Chemistry of Fats and Other Lipids*, vol. 3, p. 275. London and New York: Pergamon Press.
Shorland, F. B. (1956). *Food Manuf.* **31**, 272.
Shorland, F. B. & De La Mare, P. B. D. (1945). *J. agric. Sci.* **35**, 33.
Shorland, F. B., Weenink, R. O. & Johns, A. T. (1955). *Nature, Lond.*, **175**, 1129.
Society of Public Analysts (1933). Report of the Sub-Committee on the Determination of Unsaponifiable Matter in Oils and Fats, *Analyst*, **58**, 203.
Swern, D. & Parker, W. E. (1953). *J. Amer. Oil Chem. Soc.* **30**, 5.
Warner, A. C. I. (1956). *J. gen. Microbiol.* **14**, 733.
Weenink, R. O. (1956). *Nature, Lond.*, **178**, 646.
Willey, N. B., Riggs, J. K., Colby, R. W., Butler, O. D. & Reiser, R. (1952). *J. Anim. Sci.* **11**, 705.

The Ninhydrin Reaction and its Analytical Applications

By H. MEYER
Department of Biological and Colloid Chemistry, The Hebrew University, Jerusalem, Israel

(*Received* 25 *January* 1957)

The colorimetric or spectrophotometric measurement of the absorption produced by the purple reaction product (ANP) between α-amino acids and ninhydrin at 570 mμ has become one of the most widely used methods for the quantitative determination of small quantities of α-amino acids. Its analytical application is, however, sometimes adversely affected by one or more of the following factors. Since the ANP has not been prepared in the solid state, its extinction coefficients could not be determined; in consequence it has been necessary to express analytical results in terms of 'colour yield' in relation to that given by known quantities of a reference standard reacting under defined conditions (Moore & Stein, 1948). It has often been observed that the ANP yield was not constant owing to the occurrence of oxidative side reactions decomposing it to colourless products. Moore & Stein (1948) sought to overcome this effect by the addition of reducing agents like stannous chloride or hydrindantin to the reaction systems. The absorption maximum of the ANP was found to vary within the 500–580 mμ range, thus making impossible absorption measurements at a fixed wavelength. A method devised to eliminate this effect, by treating with alkali spots of ANP on chromatograms, was described by Kay, Harris & Entenman (1956).

The sensitivity of the reaction, i.e. the colour yield by a given amino compound produced under defined conditions, is significantly influenced by the composition of the solvent in which the reaction takes place. It is highest in organic solvents and decreases in solvent mixtures containing water (Yanari, 1956).

The experiments reported in this paper were designed to investigate the properties and behaviour of ANP, especially its absorption spectrum and its changes under different conditions and to correlate the changes in ninhydrin reactivity with measurable properties of this compound and, in turn, with structural changes undergone by the ninhydrin molecule in different solvents.

MATERIALS AND METHODS

All chemicals used were of analytical grade. The disodium salt of ethylenediaminetetra-acetic acid (EDTA) was recrystallized from water before use. Hydrindantin was

prepared according to West & Rinehart (1942) by reduction of ninhydrin with ascorbic acid.

Solvents. The absorption spectra of ninhydrin and hydrindantin were measured in absolute ethanol. In all other experiments 95·5% (w/v) ethanol was used. All water used was twice distilled. Spectrophotometric measurements were performed in a Beckman quartz spectrophotometer model DU, in 1 cm. cells.

Preparation of ANP. A solution of 0·5 g. of DL-alanine, 0·2 g. of ninhydrin hydrate and 0·1 g. of EDTA in 50 ml. of water was refluxed for 8 min. and cooled to room temp. Small quantities of bis-diketoindanyl (Moubasher & Ibrahim, 1949) were filtered off. The filtrate was then cooled to 2° and acidified with cold (2°) 20% (w/v) H_2SO_4. The reddish precipitate formed was filtered off and washed with ice-cold water as rapidly as possible, until the filtrate showed a strong purple colour. The precipitate was then sucked dry and suitable fractions of the still slightly wet material were taken for nitrogen determination and spectrophotometric measurements. Another weighed portion was dried to constant weight at 105°. All analytical results were calculated with this dry-weight determination. The nitrogen contents of a wet sample, recalculated to dry weight, and that determined after previous drying were identical, so that no loss of material had occurred on drying (Found: N, 4·5; 4·7. $C_{18}H_9O_4N$ requires 4·6%). It was, however, found that a loss of 10–12% of absorption occurred on drying, indicating that a colourless non-volatile substance had begun to form, presumably hydrindantin (Moubasher & Ibrahim, 1949). The absorption spectrum is shown in Fig. 1.

Ninhydrin blue. A solution of the blue compound formed by ninhydrin with concentrated alkali (ninhydrin blue) suitable for spectrophotometric measurements was prepared by dissolving 13 mg. of ninhydrin hydrate in 3 ml. of 6N-NaOH. A blue colour is rapidly formed which is stable to air and heating, but disappears when the solution is diluted with water.

Hydrindantin blue. A solution of the blue compound formed by hydrindantin with alkali (hydrindantin blue) was prepared by dissolving 10 mg. of hydrindantin in 25 ml. of 0·1N-NaOH. For spectrophotometric measurements the solution was rapidly transferred to the absorption cell and covered with a layer of liquid paraffin to protect it from air.

General procedure. The reaction mixture, consisting of 0·1 ml. of an aqueous solution of the nitrogen compound to be tested, 0·2 ml. of 0·3% aq. EDTA soln. and 4 ml. of 0·2% ninhydrin soln. in the solvent employed in the respective experiment was refluxed for 20 min. in a 25 mm. × 200 mm. Pyrex test tube fitted with a cold-finger condenser reaching to within 3 cm. above the boiling solution. The cooled solution was filtered, if necessary, from crystals of EDTA through a small wad of cotton wool and measured in the spectrophotometer at 578 or 408 mμ, if necessary after dilution with an appropriate volume of solvent. The reagent blank produced by ninhydrin with recrystallized EDTA is negligible when measurements are taken at 578 mμ, but at 408 mμ the reagent blank is significant owing to the absorption of ninhydrin at this wavelength (see Fig. 2).

For experiments in which the dependence of ninhydrin reactivity with amino acids upon the pH of the reaction mixture was determined: reaction mixtures of constant ninhydrin content and varying pH were prepared by bringing 25 ml. of a 0·4% aq. ninhydrin soln. to approximately the desired pH by the addition of 0·1N-HCl or NaOH and adding water to make the total volume 50 ml. The final pH of 4 ml. of this solution, to which 0·1 ml. of water containing 500 μg. of DL-alanine but no EDTA was added, was then accurately determined.

For experiments to test the availability of the 'general procedure' for the quantitative determination of amino nitrogen 95·5% (w/v) ethanol was used as the solvent.

(*a*) Determinations of colour yield at 578 and 408 mμ at different concentrations were carried out for all the natural amino acids and glycylglycine, glycyl-leucine, leucylglycine, triglycine and leucylglycylglycine. They were added as aqueous solutions containing from 0·2 to 9 μg. of α-amino nitrogen/0·1 ml.

(*b*) The influence of buffers normally used was determined in a parallel series of experiments, under the same conditions except that the amino acids and peptides were dissolved in acetate, or citrate or phosphate buffers (0·1M, pH 6·5).

(*c*) The reactivity of nitrogen-containing buffers was tested by adding 5 mg. of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) or sodium diethylbarbiturate in 0·1 ml. of water to a reaction mixture not containing other nitrogen compounds.

(*d*) Ninhydrin reactivity with free ammonia, methylamine, ethylamine and ethylenediamine was tested in reaction mixtures containing quantities of each nitrogen compound equivalent to 10 μg. of nitrogen. Ammonium chloride and the hydrochlorides of the amines mentioned were added in quantities equivalent to 2 mg. of nitrogen.

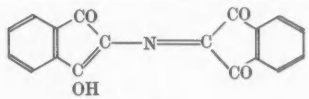
All experiments mentioned under (*a*) and (*d*) were also carried out with hydrindantin instead of ninhydrin.

Kinetic experiments. The desired amount of DL-alanine was dissolved in 4 ml. of water, 8 ml. of a 0·3% aq. EDTA soln. was added, followed by 160 ml. of ethanol containing the desired amount of ninhydrin. This reaction mixture was brought to the boil in a cylindrical separating funnel of 250 ml. capacity, externally wound with electrical-heating tape and fitted with a reflux condenser. The reaction mixture was stirred by a vigorous stream of nitrogen gas. Samples (3 ml.) were withdrawn through the tap of the funnel into test tubes kept in ice–water at 20 sec. intervals during the first 2 min. of reaction time (counting from the beginning of boiling), at 30 sec. intervals during the following 6 min., and then every 3 min. until the end of the reaction time (50 min.). Spectrophotometric measurements were taken at 578 and 408 mμ; if necessary, the samples were diluted with an equal volume of ethanol. All reactant concentrations are given in Table 1.

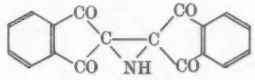
RESULTS

Properties of ANP. In the solid state ANP is a reddish substance, easily soluble in all solvents containing alcoholic or phenolic hydroxyl groups and insoluble in ether, chloroform and non-polar organic solvents. Attempts to recrystallize it from water failed owing to decomposition on warming. It completely decomposes in dilute aqueous solution if kept for some hours, and almost immediately when such solutions are acidified and slightly warmed. Alkaline ANP solutions and those containing 0·1% of EDTA are stable for some days. Solutions of purified ANP in ethanol or *n*-butanol, stabilized by the addition of EDTA, can be kept for

several weeks. A characteristic property of ANP, which can also be used to purify the compound, is its specific absorption by anion exchangers. The effect can easily be demonstrated by shaking aqueous ANP solutions with De-Acidite or Amberlite IR-4 B in their OH^- forms. Elution can be effected with aq. N-NaCl soln. or with ethanol. The nitrogen content of the ANP fits the structures proposed by Ruheman (1911) (I) and by Woker & Antener (1937) (II). Both (I) and (II) are assumed to be anionic structures (Clarke, 1948; Wieland, 1949).



(I)



(II)

The absorption spectrum of pure ANP shows two well-defined bands, with $\lambda_{max.}$ 578 and 408 mμ and $E^{1\%}_{1\,cm.}$ 660 and 724 respectively (Fig. 1, curve 2). The maximum at 408 mμ is especially characteristic for ANP, since it does not appear in the absorption spectra of the nitrogen-free compounds ninhydrin blue and hydrindantin blue (Fig. 5) which both absorb strongly in the 570–580 mμ region. The absorption maximum at 408 mμ does not form a part of the absorption spectrum of murexide, which is sometimes regarded (Clarke, 1948) as a structural analogue of ANP (Fig. 1, curve 3).

Since the extinction coefficients of ANP have been determined, it can be decided whether ANP contains all the nitrogen originally present in the amino compound from which it had been formed. A solution of 9·2 mg. of ANP/l. gave $E_{1\,cm.}$ at 578 mμ, 0·600. The same extinction was measured in an ANP solution in which the ANP had been formed by the reaction of 11·62 μg. of alanine with ninhydrin. The reaction mixture (total vol. 4·3 ml.) contained therefore 1·83 μg. of amino nitrogen, which corresponds, calculated to a nitrogen content of the ANP of 4·62 %, to an ANP concentration of 0·2 mg./l. All the alanine nitrogen has therefore been converted into ANP nitrogen.

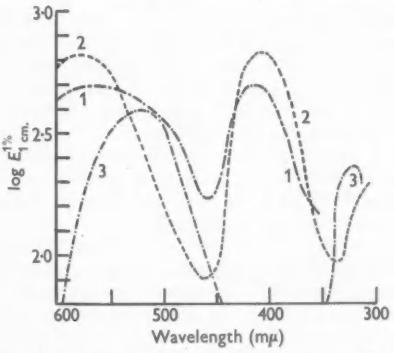


Fig. 1. Absorption spectra: 1, ANP (10 mg./l.) in *n*-butanol; 2, ANP (10 mg./l.) in ethanol or water, containing 1 % (v/v) of 0·3 % aq. EDTA soln.; 3, murexide (10 mg./l.) in water.

The absorption spectrum of ANP formed in a reaction mixture in *n*-butanol in the complete absence of water is shown in Fig. 1, curve 1. It can be seen that absorption at both characteristic wavelengths is depressed, but enhanced at all others up to 530 mμ. The colour of the material produced in butanol is reddish instead of the deep-purplish blue. When minute amounts of water are present in the reaction mixture, absorption spectra are produced which vary in appearance between the extreme shown and that of the normal ANP spectrum. When the water content of the reaction mixture reaches or exceeds 5 %, the normal ANP spectrum is always produced. Since the existence of an anionic structure in the ninhydrin ANP molecule is probable, it seems reasonable to assume that the normal ANP spectrum is due to the existence of the ANP anion, whereas the spectra produced in the absence of water are characteristic of the undissociated form of ANP.

Kinetics of the formation of ANP. The results of the kinetic experiments are given in Table 1. Colour formation was preceded by a latent period during which no colour was formed. The colour-formation reaction itself, when measured at any of the ninhydrin concentrations given in Table 1 and at alanine concentrations varying from 0·0129 to 0·103 m-mole/l., followed (within an experimental error of 5 %) unimolecular kinetics, the rate of ANP formation being independent of the initial alanine concentration. Measurements taken at constant alanine and varying ninhydrin concentrations showed, however, that both the latent periods and the reaction half-times were not independent of

Table 1. *Kinetic data on the formation of ANP by the reaction of ninhydrin with* DL-*alanine*

Alanine concentration was 0·0258 m-mole/l.

Concn. of ninhydrin (m-moles/l.)	Molar concn. ratio (alanine:ninhydrin)	Latent period (sec.)	Reaction half-time (min.)
5·82	1:225	61	5
11·64	1:450	30	2·5
17·76	1:675	15	2·0
22·38	1:900	6	1·7
56·56	1:1800	6	1·7

ninhydrin concentration, as should be expected with the large excesses of ninhydrin used (see Table 1). It can be seen that both latent period and reaction half-time become independent of ninhydrin concentration only when the molar-concentration ratio alanine:ninhydrin equals or exceeds 1:900. This result seems to show that, of the total ninhydrin concentration present, only a small part is available for colour formation.

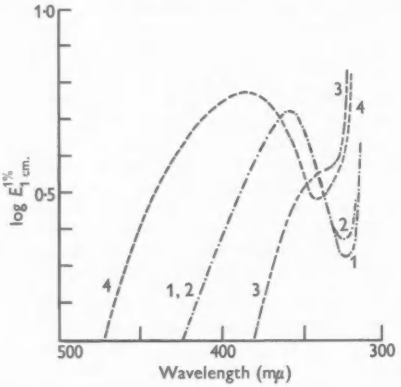


Fig. 2. Absorption spectra: 1, ninhydrin (1 g./l.) in absolute ethanol; 2, hydrindantin (1 g./l.) in absolute ethanol; 3, ninhydrin (1 g./l.) in water; 4, ninhydrin (1 g./l.) in aq. NaOH soln. (pH 10·25).

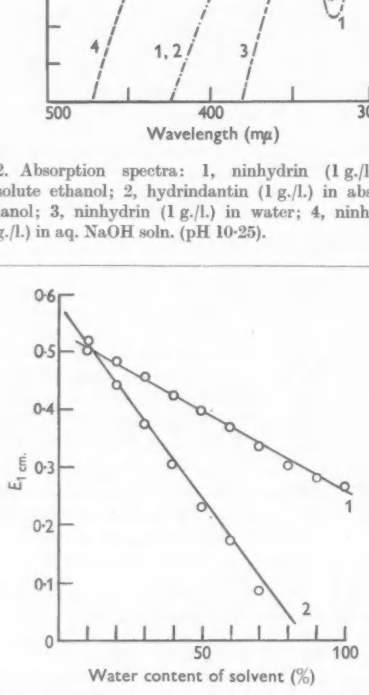


Fig. 3. Changes of absorption of ninhydrin at 356 mμ (1) and of the colour value of the product of its reaction with amino acids (2) with increasing water content of the solvent. For (2), 10 μg. of DL-alanine was used in 0·2% ninhydrin soln.; other conditions are as described under Materials and Methods.

Factors affecting the reactivity of ninhydrin. Success in applying the ninhydrin reaction to analytical problems depends to a large measure upon the state of this compound in solution, since the sensitivity of the reaction is much greater in organic solvents than in water. Another factor greatly affecting sensitivity is the H^+ ion concentration (McFadyen, 1948), especially in aqueous solution. As ninhydrin solutions are known to deteriorate with time, a method of standardizing such solutions to constant activity would be desirable.

The following experiments were an attempt to provide evidence for the existence of an active form of ninhydrin, the concentration of which varies with different conditions and determines the sensitivity of the ninhydrin reaction. It was found that reactivity changes of ninhydrin, especially those occurring upon changing the composition of the solvent, can be correlated with changes in its absorption spectrum happening at the same time. The absorption spectrum of ninhydrin in ethanol is shown in Fig. 2, curve 1. The absorption maximum at 356 mμ almost completely disappears when the solvent is water (Fig. 2, curve 3). When absorption at 356 mμ was measured in ninhydrin solutions where the ethanol was gradually replaced by water, it could be shown that the decline in absorption is linearly proportional to the increase in water content (Fig. 3, curve 1). Concurrently, the colour yield obtained with alanine also declined in linear proportion with increasing water content of the solvent (Fig. 3, curve 2).

Since both absorption at 356 mμ and the ANP-producing activity of ninhydrin are linearly proportional to the water content of the solvent, a linear-functional relationship exists between the two factors. The colour-producing activity of ninhydrin in any solvent mixture can therefore be expressed in terms of the extinction coefficient measured at 356 mμ. Ninhydrin solutions can thus be standardized to their maximum or any given percentage activity, either by the addition of water to the ethanolic ninhydrin solutions or by varying the ninhydrin concentration.

The 0·2% ninhydrin soln. in ethanol generally used in the experiments reported in this paper was standardized to an extinction value of 1·00 at 356 mμ, measured in a 1 cm. cell.

Apart from the composition of the solvent, the sensitivity of the ninhydrin reaction is most influenced by the H^+ ion concentration in the solvent. In aqueous solutions with a pH of below 4 no colour formation took place. Reactivity, as expressed by colour yield, increases sharply to reach its peak at pH 6·18, declined then to half its maximum value at pH 8·8 and disappeared completely at pH 10 and above (Fig. 4, curve 1). The absence of reactivity in

acid solutions is due to the instability of ANP in acid. When solutions containing ANP were acidified, the ANP colour disappeared almost instantaneously in solutions of pH of below 4 and more slowly in less acid solutions. It can therefore be concluded that in such solutions the colour yield observed depends on the ratio between the rates of formation and destruction of ANP. No such effect is possible in alkaline solutions in which ANP is stable; reactivity changes in alkaline solutions have therefore to be explained by other effects, such as changes in the ninhydrin molecule. Evidence for such an effect can be seen in the significant changes occurring in the ninhydrin-absorption spectrum in alkaline solution, in contrast with acid conditions where no changes in the absorption spectrum of ninhydrin could be detected at different degrees of acidity. A ninhydrin solution in water to which increasing quantities of alkali are added turns yellow and shows a new absorption maximum at 380 mμ. Extinction at this wavelength reached half its maximum value at pH 8·8 and its maximum value at pH 10·25 (Fig. 4, curve 2). (The shape of the absorption spectrum at this point is shown in Fig. 2, curve 4.) Extinction at 380 mμ declines then rapidly and disappears completely at pH 12 and higher.

The potentiometric titration curve of ninhydrin (Fig. 5) shows that ninhydrin behaves like an acid with a pK of 8·8. When the alkali concentration of aqueous solutions containing ninhydrin is increased to 6N, the colour of ninhydrin blue appears. Its absorption spectrum (Fig. 6) shows, in addition to the maximum at 580 mμ, a pronounced absorption maximum at 356 mμ resembling that shown by ninhydrin in ethanol (Fig. 2, curve 1). It should be noted that the absorption spectrum of ninhydrin blue is very similar to that of hydrindantin blue, a compound formed when hydrindantin, the reduction product of ninhydrin, is dissolved in 0·1N-NaOH (Fig. 6).

Hydrindantin has been shown to react with amino acids in an aqueous medium and to yield the same reaction products, including ANP (Moubasher & Ibrahim, 1949). We found that in ethanolic solution hydrindantin produces amounts of ANP equal to those obtained with ninhydrin under similar conditions.

Quantitative determination of amino acids

Complete conversion of the α-amino nitrogen into ANP nitrogen, i.e. a theoretical colour yield, was obtained with all the amino acids and peptides

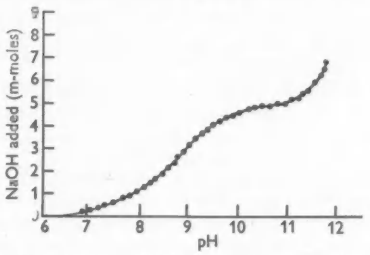


Fig. 5. Titration curve of ninhydrin (4·92 m-moles titrated).

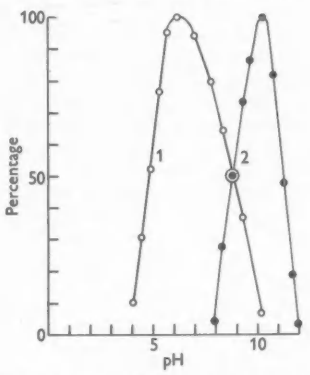


Fig. 4. Changes in the colour-producing activity of ninhydrin with amino acids (1) and of its absorption at 380 mμ (2) with pH in aqueous solution, expressed as percentages of the respective maximum values. Absorption of ninhydrin was measured in 0·2% soln. and colour values were determined with 500 μg. of DL-alanine in 0·2% aq. ninhydrin soln.; other conditions as described under Materials and Methods.

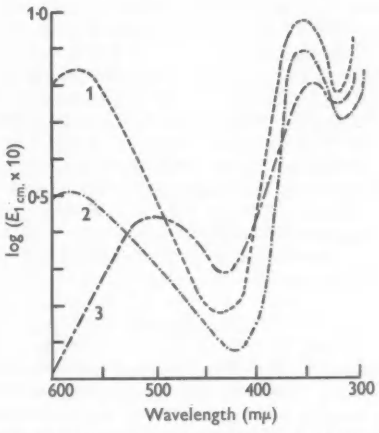


Fig. 6. Absorption spectra: 1, hydrindantin blue (from 10 mg. of hydrindantin in 25 ml. of 0·1N-NaOH); 2, ninhydrin blue (from 13 mg. of ninhydrin in 3 ml. of 6N-NaOH); 3, hydrindantin in ethanol (1 g./l., freshly prepared solution).

tested, with the exception of proline, hydroxyproline, phenylalanine, tyrosine and tryptophan. Hydroxyproline and proline cannot be determined by any method measuring ANP production, since their reaction product with ninhydrin differs in structure and absorption characteristics from ANP (Grassmann & Arnim, 1934). Phenylalanine, tyrosine and tryptophan yield about 70% of the theoretically expected colour value. They can, however, be determined with the use of special calibration curves, since the colour values given by them were reproducible. In all other cases the construction of a single calibration curve is sufficient, or the α-amino nitrogen content of the sample analysed can be calculated directly by

$$\mu\text{g. of } \alpha\text{-amino nitrogen in sample} = 100Avu/(E^{1\%}_{1\,\text{cm.}} \times l),$$

where A is the extinction (optical density) value measured for the sample, v is the total volume of the reaction mixture (4·3 ml.), u is the nitrogen content of ANP (in %), $E^{1\%}_{1\,\text{cm.}}$ is the specific extinction coefficient of ANP at the wavelength used and l is the cell length in cm.

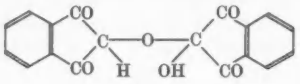
It should be noted that the method is strictly specific for α-amino nitrogen; lysine, arginine, ornithine, citrulline and histidine were all found to react with their α-amino groups only.

Strictly linear dependence of extinction (optical density) on concentration was observed with samples containing amounts of α-amino nitrogen ranging from 0·5 to 6 μg. The accuracy of the method is ± 3%. The same results were obtained when the compounds tested were dissolved in acetate, citrate or phosphate buffers. The nitrogen-containing buffers tris and diethylbarbituric acid did not react with ninhydrin.

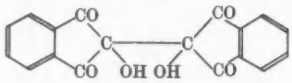
Both ammonia and the amines tested, when present as free bases, reacted in ethanolic solution with ninhydrin. They yielded 17–30% of the amount of ANP theoretically expected, but the results obtained varied within very wide limits. The interference of ammonia and amines with the determination of amino acids can, however, easily be eliminated by converting them into their salts, since ammonium and amine salts did not yield measurable amounts of colour.

DISCUSSION

Structure of ninhydrin. Since it has been shown (Fig. 2) that the absorption spectra of ninhydrin and hydrindantin are the same in the region most connected with activity changes, it may be concluded that both compounds owe their reactivity with amino compounds to similar structures. According to its discoverer, Ruheman (1911), hydrindantin is a condensation product of ninhydrin (V) with its reduction product 1:3-dioxo-2-hydroxyindane and has the structure (III). Schönberg & Moubasher (1949) favoured structure (IV). Hydrindantin does

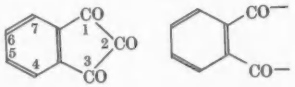


(III)



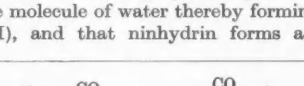
(IV)

not therefore contain the carbonyl group in the 2-position to which the reactivity of ninhydrin (V) with amino compounds is generally believed to be due (Clarke, 1948). It is therefore difficult to see how hydrindantin could react with amino compounds at all, and why ninhydrin, usually represented as (V), should show exactly the same absorption characteristics in the 300–400 mμ region as its reduction product, hydrindantin.

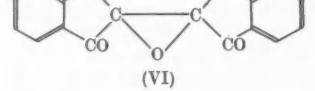


(V) (V*a*)

The system (V*a*), common to both hydrindantin and ninhydrin, cannot be responsible for this effect since the absorption spectrum of ANP, which also contains (V*a*), does not show any absorption maximum at 356 mμ. It is therefore assumed that hydrindantin, on dissolving in organic solvents, loses one molecule of water thereby forming structure (VI), and that ninhydrin forms a similar



(VI)

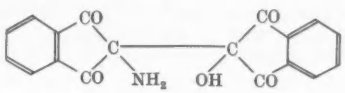


(VII)

structure (VII) by losing two molecules of water. This structural change is in hydrindantin accompanied by absorption changes. Freshly prepared hydrindantin solutions show a red colour with an

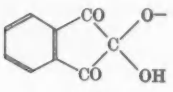
absorption maximum at 510 mμ (Fig. 6, curve 3). When kept the colour fades and the absorption spectrum then assumes the form shown in Fig. 2, curve 2. Absorption at 356 mμ is due to the presence of (VI) and (VII), which are the reactive forms of hydrindantin and ninhydrin respectively, but appear in solution only in small equilibrium concentrations. This assumption is borne out by the fact that a very large excess of hydrindantin and of ninhydrin is necessary to make them react with amino compounds. The dependence of both absorption at 356 mμ and reactivity of ninhydrin become now easily explainable by assuming that, in the presence of water, (VII) is split into two molecules of (non-reactive) ninhydrin hydrate. Evidence for the existence of a condensed form of ninhydrin can be seen in the fact that hydrindantin has been prepared by Schönberg & Moubasher (1949) by the action of ninhydrin and magnesium in the presence of magnesium iodide in ether. This is essentially the method for the synthesis of pinacols from aromatic ketones published by Gomberg & Bachmann (1927), which is believed (Wheland, 1953) to lead through the intermediate stage of the often highly coloured metal ketyls. It is suggested that the deep-blue compounds ninhydrin blue and hydrindantin blue both formed by the action of alkali on the parent compounds could be regarded as analogous to the metal ketyls. The fact that ninhydrin blue has an absorption spectrum very similar to that of hydrindantin blue (Fig. 6) can also be taken as evidence for the existence of a condensed form of ninhydrin, since hydrindantin blue is most probably to be regarded as a derivative of the condensed structure (IV).

Simple reaction mechanisms for explaining the ninhydrin reaction, the reactivity of amino compounds with ninhydrin and hydrindantin, can be constructed with the use of structures (VI) and (VII), the complete oxidative deamination of the amino compounds being performed by one molecule of ninhydrin or two molecules of hydrindantin, leading to the common intermediate (VIII) from which either (I) or (II) could be formed.

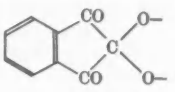


(VIII)

Structure of ninhydrin in aqueous solution. The greatly diminished reactivity of ninhydrin in aqueous solution, due to the prevalence of the non-reactive form (V), is still further reduced and finally abolished when such solutions are made alkaline (Fig. 4). This effect can be explained by the assumption that ninhydrin (V), which in aqueous solution can still form a small amount of the active form (VII) of ninhydrin, can not do so if converted by alkali into the univalent ion (IX). This ion is



(IX)



(X)

capable of forming resonance structures and therefore shows absorption (at 380 mμ). At pH 8·8, the pK of the acid form of ninhydrin, ninhydrin reactivity has half the value it has under conditions excluding the presence of (IX). Concurrently, absorption at 380 mμ, which is due to the formation of (IX), has half its maximal value at the same pH. At pH 10·25 all ninhydrin has been converted into (IX); accordingly, absorption at 380 mμ is maximum and reactivity is completely abolished. When the solution is made still more alkaline, the bivalent ion (X) is formed which is incapable of forming resonance structures. There is then no absorption at 380 mμ (Fig. 4).

Analytical conclusions

The high sensitivity of the ninhydrin reaction can be fully utilized when ethanol is used as the solvent, since in ethanol the formation of the active form of ninhydrin (VII) is favoured and the formation of its inactive forms (V), (IX) and (X) is largely suppressed. Sensitivity is not measurably diminished by the amount of water present, which is necessary to avoid the formation of atypical ANP spectra and to hold a sufficient amount of EDTA in solution. Ethanol also largely obviates any interference likely to be caused by the pH of the solution to be analysed, except in extreme cases. Solutions containing the amino acid to be determined may vary from pH 4·5 to 8. When ammonia or amines are present, care should be taken to neutralize them completely.

Interfering factors. Strong oxidizing or reducing substances destroy the ANP. Trichloroacetic acid seriously interferes with ANP formation and must therefore quantitatively be removed by ether extraction from solution to be analysed. The interference caused by metal ions present in the concentrations usually found in biological systems is eliminated by the use of EDTA. No other interfering factors have so far been found.

The method described is primarily intended for the determination of amino acids and peptides in column eluates or cell extracts. It can be adapted to the determination of amino acids on paper chromatograms; the zones containing the amino

acids are cut out and refluxed in a reaction mixture consisting of 4 ml. of ninhydrin solution and 0·2 ml. of EDTA solution and 0·1 ml. of water.

The method has been so far successfully employed for the determination of amino acids in ether-treated trichloroacetic acid and warm-water extracts of yeast cells (Benziman, 1956) and for following the enzymic decomposition of polylysin (M. Rigbi, personal communication).

SUMMARY

1. The coloured reaction product of amino acids with ninhydrin (ANP) was prepared in the solid state. Extinction coefficients for its absorption maxima at 578 and 408 mμ were determined.

2. By using the fact that ANP is greatly stabilized in the presence of ethylenediaminetetra-acetic acid, uniform absorption spectra are obtained and oxidative side reactions leading to the decomposition of ANP are avoided.

3. The α-amino nitrogen of amino acids is quantitatively transferred to ANP.

4. The changes in the reactivity of ninhydrin with amino compounds in different ethanol–water mixtures and in aqueous solutions at different H^+ ion concentrations, and the accompanying changes in its absorption spectrum, are explained by structural changes in the ninhydrin molecule. A structure for the reactive form of ninhydrin and hydrindantin is suggested.

5. An analytical method for the spectrophotometric determination of 0·5–6 μg. of α-amino nitrogen, contained in amino acids or peptides, is described.

The author wishes to express his gratitude to Professor A. Neuberger, F.R.S., for helpful advice and discussions.

REFERENCES

Benziman, M. (1956). Ph.D. Thesis: The Hebrew University, Jerusalem.
Clarke, H. T. (1948). *Organic Chemistry*, 2nd ed., vol. 2, p. 1049. Ed. by Gilman, H. New York: Wiley.
Gomberg, M. & Bachmann, W. E. (1927). *J. Amer. chem. Soc.* **49**, 236.
Grassmann, W. & Arnim, K. v. (1934). *Liebigs Ann.* **509**, 288.
Kay, R. E., Harris, D. H. & Entenman, C. (1956). *Arch. Biochem. Biophys.* **63**, 14.
McFadyen, D. A. (1948). *J. biol. Chem.* **153**, 507.
Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
Moubasher, R. & Ibrahim, J. (1949). *J. chem. Soc.* p. 702.
Ruheman, J. (1911). *J. chem. Soc.* **99**, 797.
Schönberg, A. & Moubasher, R. (1949). *J. chem. Soc.* p. 212.
West, E. S. & Rinehart, R. E. (1942). *J. biol. Chem.* **146**, 105.
Wheland, G. W. (1953). *Advances in Organic Chemistry*, 2nd ed., p. 717. New York: Wiley.
Wieland, T. (1949). *Fortschr. chem. Forsch.* **1**, 211.
Woker, G. & Antener, I. (1937). *Helv. chim. acta*, **20**, 1260.
Yanari, S. (1956). *J. biol. Chem.* **220**, 683.

Blood Lipids

1. PLASMA LIPIDS OF THE LACTATING COW: CHROMATOGRAPHY ON SILICIC ACID

By G. A. GARTON and W. R. H. DUNCAN
Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 13 *March* 1957)

Arteriovenous studies (for review see Folley, 1956) have demonstrated that no component of blood lipids except triglycerides and possibly cholesterol esters is absorbed from the blood plasma by the lactating ruminant udder. It is therefore of some importance to study the fatty acid composition of these classes of lipid, though it is first necessary to separate them from each other and from other plasma lipids.

The separation of mixtures of lipids into their component classes was restricted for many years to their segregation into acetone-insoluble lipids (phospholipids) and acetone-soluble lipids (glycerides, sterol esters, free sterols and fatty acids), from which mixture the last two components could each be recovered chemically. However, no means of distinguishing between fatty acids in combination as glycerides and as sterol esters was devised until Kelsey (1939) used pig pancreatic lipase for the selective hydrolysis of glycerides in the presence of cholesterol esters. This procedure was employed by Kelsey & Longenecker (1941) in an investigation of the component fatty acids of the plasma lipids of lactating cows. As has been discussed by Lovern (1956), it seems that the enzymic procedure is unsatisfactory in that it does not effect the complete hydrolysis of glycerides. Clément, Clément-Champougny & Louedec (1954) found that the method given by Kelsey (1939) did not effect more than 67 % hydrolysis of the glycerides from various

animal sources, including dog and rabbit serum. This finding is in accord with the studies of Desnuelles, Naudet & Rouzier (1948) and Savary & Desnuelles (1956) on the hydrolysis of triglycerides by pig pancreatic lipase *in vitro*.

In the present study the more recently developed technique of adsorption on silicic acid followed by selective elution (Fillerup & Mead, 1953*a*) was successfully employed to separate the classes of lipids present in extracts of the plasma of lactating cows with particular reference to the sterol esters and triglycerides. A preliminary account of part of this work has been published (Lough, Garton & Duncan, 1957).

EXPERIMENTAL

Solvents. Absolute ethanol (B.P. 1948) and methanol (A.R.) were used as supplied by J. Burrough Ltd., London, S.E. 11. Light petroleum (b.p. 40–60°) was of A.R. grade (British Drug Houses Ltd.). Ether was dried and rendered free from peroxides immediately before use by distillation over KOH and reduced iron.

Analytical methods. Iodine values were determined by the semi-micro method of Trappe (1938). Phosphorus was estimated according to Berenblum & Chain (1938) after wet-ashing (with H_2SO_4) of the lipid; lipid P was satisfactorily estimated in egg lecithin with this ashing procedure. A solution of the lipid in $CHCl_3$ was taken for the spectrophotometric determination of carotene, the reference value $E^{1\%}_{1\,cm.}$ at 463 mμ = 2200 (Morton, 1942) being used. Free fatty acids, and possibly other acidic lipids, were estimated by titration of a solution of lipid in ethanol–benzene (1:1, v/v) against methanolic 0·01 N-KOH, with bromothymol blue as indicator. Titration was difficult owing to the orange colour of the solutions, and the values given (Table 1) are approximations, erring on the high side. Free and ester cholesterol were determined essentially according to Schoenheimer & Sperry (1934); satisfactory recovery of authentic cholesterol and cholesterol from cholesterol palmitate was obtained. Glycerol was determined by the method of Lambert & Neish (1950) as modified by Korn (1955); glyceride fatty acids were determined by titration of the acids isolated from hydrolysates.

Blood samples. Blood was obtained at the Aberdeen slaughterhouse from the jugular vein of a 3-year-old lactating Ayrshire cow and by jugular venepuncture from each of six lactating Ayrshire cows (aged 3–13 years) of the Duthie Experimental Stock Farm herd of this Institute. The blood was taken into saturated sodium citrate solution (20 ml./l. of whole blood) and centrifuged as soon as possible to obtain the plasma. From the slaughtered animal, subsequently referred to as cow 1, 2740 ml. of plasma was obtained. From each of the living animals (cows 2–7) about 500 ml. of plasma was obtained; before further treatment the plasma was pooled to give a total of 2740 ml. The plasma volumes given above have been 'corrected' for their content of citrate solution.

Diets of cows. Cow 1, which had been fasting for 12 hr. when the blood sample was taken, was previously fed on a diet consisting mainly of turnips, silage and concentrates. Cows 2–7 had been pasture-fed for 4 weeks.

Treatment of plasma. The plasma was poured slowly, with shaking, into 2 vol. of ethanol and allowed to stand for 1–2 hr. at room temperature. The precipitated protein (containing most of the lipids) was filtered and the filtrate concentrated to about 250 ml. *in vacuo* in an atmosphere of N_2. The concentrate was extracted with 3 × 100 ml. of light petroleum–ether (1:1, v/v). The extract was dried over anhydrous Na_2SO_4, filtered and combined with the lipid-containing extracts of the precipitated protein (see below).

The protein 'cake' was dispersed in ethanol–ether (3:1, v/v) in the proportion of 1 l. of solvent/l. of original plasma. The mixture was refluxed on a water bath for 4 hr. and then filtered at the pump. Extraction of the protein was repeated twice and after the third extraction the protein 'cake' was dispersed in hot ethanol–ether and thoroughly washed on the filter with hot solvent. The combined ethanol–ether extracts were concentrated to about 250 ml. on a water bath under reduced pressure in an atmosphere of N_2. At this stage the concentrated extract became cloudy, suggesting that water present was rendering some of the lipid insoluble. The concentrated extract (lipids plus aq. ethanol) was extracted with 6 × 100 ml. of light petroleum and the solution was dried over anhydrous Na_2SO_4. After filtration most of the solvent was removed before the extract was combined with the light petroleum–ether solution of lipid derived, as explained above, from the aq. ethanol filtrate of the precipitated plasma proteins. The solvents were finally removed to yield, in each case, a viscous, orange–red oil which was stored under N_2 at −1° in the dark for a few days until the chromatographic examination was begun. The yields of lipid and some of their analytical characteristics are shown in Table 1. No water-soluble free fatty acid was present in either of the lipid preparations.

Preliminary experiments on chromatographic separation of lipids. Before silicic acid of American origin, as used by Fillerup & Mead (1953*a*, *b*), Mead & Fillerup (1954) and Borgström (1952*a*, *b*), was successfully employed (see below) several silica-gel preparations were examined in conjunction with various eluting solvents, including those recommended by Mead & Fillerup (1954). The silica-gel samples used were (1) prepared from water glass in 1947 by Dr R. L. M. Synge in the same way as described for silica gel 'ϵ' (Gordon, Martin & Synge, 1944); (2) made by Dr P. J. Heald in 1950 from water glass according to Gordon, Martin & Synge (1943); (3) prepared from 'silicic acid' (British Drug Houses Ltd.) by heating at 120° for 24 hr. followed by grinding and sieving through a 160-mesh B.S. sieve. Slurries in light petroleum, prepared from gels 1 and 2 before and after heating at 120° for 24 hr., and from gel 3, were introduced into glass tubes to form columns 7 cm. × 2·5 cm. Silica gel 3 and gel 2 before or after heat treatment

Table 1. *Amounts of plasma lipids extracted and their general analytical characteristics*

	Cow 1 (2740 ml.)	Cows 2–7 (2740 ml.)
Lipids extracted (g.)	6·07	6·69
Lipids (mg./100 ml. of plasma)	221	244
Iodine value	99·0	115·5
Acidity (as oleic acid, %)	1·9	0·9
Carotene (μg./100 ml. of plasma)	616	1216
Phosphorus (mg./100 g. of lipid)	7	82

offered considerable resistance to the flow of light petroleum or methanol even when 'positive' pressure was applied; the flow-resisting properties were not greatly altered when Hyflo Super-Cel (Johns Manville, London, S.W. 1) was previously mixed with the gels in proportions of up to 1:3 (w/w). Trials with these samples were therefore abandoned, as were trials with silica gel 1, which although it gave convenient flow rates, failed to adsorb any lipid applied in solution in light petroleum.

A small sample of silicic acid (Baker Chemical Co.) which had been heated to 120° for 24 hr. was obtained through the courtesy of Dr B. Borgström (University of Lund, Sweden) and this was found to give a suitable flow rate with light petroleum when mixed with 0·4 of its weight of Hyflo Super-Cel. When applied to the column in amounts of about 30 mg. of lipid/g. of adsorbent (cf. Borgström, 1952*b*) effective separation was achieved of artificial mixtures of two or more of the following lipids: cholesterol palmitate (British Drug Houses Ltd.), lard triglycerides [purified by crystallization at −10° from a 10% (w/v) solution in acetone], cholesterol (British Drug Houses Ltd.; m.p. 146°) and egg-yolk phospholipids (prepared by repeated acetone precipitation from fresh-yolk lipids). No lipid was eluted with light petroleum alone; light petroleum containing 1, 3 and 10% (v/v) of ether was employed to elute successively cholesterol palmitate, lard glycerides and cholesterol. Phospholipids were subsequently eluted with ether–methanol (3:1, v/v). When palmitic acid (British Drug Houses Ltd.) was included in the mixtures it could not be separated effectively from triglycerides by employing successively 3, 4 and 5% of ether in light petroleum as the eluting solvent (cf. Mead & Fillerup, 1954).

RESULTS

Chromatographic separation of plasma lipids

A large batch of silicic acid powder ('Baker Analysed' Reagent, J. T. Baker Chemical Co., Phillipsburg, U.S.A.) was obtained; before use it was thoroughly washed with hot methanol, warm ether and warm acetone and activated at 120° for 24 hr. Hyflo Super-Cel was stirred with a large volume of water and the fine suspended particles which did not settle within 1 hr. were removed by suction; it was then washed by solvents and heated as described for silicic acid. Both the silicic acid and the Hyflo Super-Cel were stored in full, sealed bottles until used. The successful preliminary experiments with this brand of silicic acid were repeated with material from the large batch. Large-scale separations were then carried out with 5·60 and 5·58 g. respectively of the plasma lipids from cow 1 and cows 2–7.

For each separation a slurry of a mixture of silicic acid (200 g.) and Hyflo Super-Cel (70 g.) was made in light petroleum and the mixture was heated to boiling on a water bath to expel dissolved gases. The slurry was rapidly transferred to a glass tube with gentle tamping to give a column 21 cm. × 5·5 cm. supported on a thin layer of cotton wool on a porcelain-filter disk. The lipid, in solution in light petroleum (20 ml.), was then carefully applied to the column with a pipette and elution with light petroleum was started, followed successively by 1, 3 and 10% (v/v) of ether in light petroleum and ending with ether–methanol (3:1, v/v). Fractions each of 750 ml. of eluate were collected and the solvent was removed under reduced pressure; each eluting solvent was used until it practically ceased to bring any further lipid off the column. As a further check on the progress of elution, the lipid fractions were submitted to the Liebermann–Burchard reaction as indicated in Fig. 1. From the lipids of cow 1, 52 column fractions were collected, and 48 from the lipids of cows 2–7.

The elution pattern was very similar in each case; that for the plasma lipids of cows 2–7 is shown in Fig. 1. The recoveries from the columns were 98·4 and 97·7% respectively for the plasma lipids of cow 1 and cows 2–7. Four main peaks were obtained from each sample of total lipids, and from the work of Fillerup & Mead (1953*a*, *b*), Mead & Fillerup (1954) and our own preliminary studies it was concluded that these represented sterol esters, glycerides (together with any free fatty acids), free sterols and phospholipids. In addition, traces of lipid, possibly hydrocarbons (cf. Lovern, 1956), were eluted with the solvent front and the sterol esters were followed off the column by very small amounts of lipid, the exact nature of which remains to be determined. Appropriate fractions were combined to give the components shown in Table 2, in which certain analytical characteristics of the lipids are also recorded.

At room temperature, fractions *A* were colourless, soft and waxy, fractions *B* were viscous, deep-red oils which crystallized on standing, fractions *C*

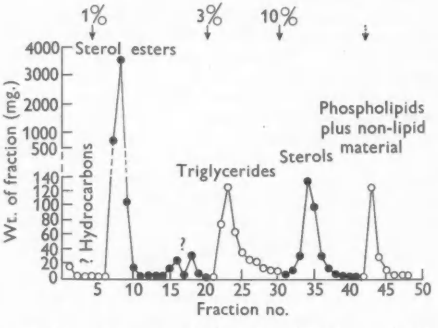


Fig. 1. Chromatography on silicic acid of plasma lipids from cows 2–7. Changes in eluting solvent (1, 3 and 10% of ether in light petroleum) are indicated by arrows; broken arrow indicates change to ether–methanol (3:1). ○, Negative Liebermann–Burchard reactions; ●, positive Liebermann–Burchard reactions.

Table 2. *Amounts of analytical characteristics of pooled chromatographic fractions*

Amounts of lipid chromatographed are given in parentheses. A dash indicates not determined, but probably negligible; (0) indicates none detected.

Designation of lipid fraction	Nature of lipid	Column fractions pooled	Wt. of lipid* (g.)	Percentage of total lipid recovered	Iodine value	Cholesterol in fraction (%)		P content (mg./100 g. of fraction)
						Free	Esterified	
			Cow 1 (7·60 g.)					
A	? Hydrocarbons	1–6	0·03	0·4	10·6	—	—	—
B	Sterol (cholesterol) esters	7–12	3·64	65·8	120·3	(0)	62·5	—
C	Contains sterol esters	13–16	0·07	1·0	93·2	(0)	43·2	—
D	Triglycerides plus a little free fatty acid	17–31	1·02	18·5	41·0	(0)	(0)	(0)
E	Sterols (cholesterol)	32–44	0·65	11·8	68·9	89·8	(0)	20
F	Phospholipids plus non-lipid material	45–52	0·14	2·5	94·5	—	—	70
			Cows 2–7 (5·58 g.)					
A	? Hydrocarbons	1–6	0·03	0·5	11·3	—	—	—
B	Sterol (cholesterol) esters	7–10	4·35	79·8	123·0	(0)	56·7	—
C	Contains sterol esters	11–20	0·11	2·0	90·0	(0)	40·9	—
D	Triglycerides	21–30	0·41	7·5	36·3	(0)	(0)	—
E	Sterols (cholesterol)	31–41	0·35	6·4	67·6	82·1	(0)	(0)
F	Phospholipids plus non-lipid material	42–48	0·20	3·8	49·7	—	—	50

* Including very small amounts used in Liebermann–Burchard tests.

formed viscous, orange–yellow oils, fractions *D* were semi-solid and virtually colourless, fractions *E* gave rosettes of pale-yellow crystals and fractions *F* formed yellow or pale-brown oils. As already mentioned, free higher fatty acids, when present, are eluted along with glycerides; accordingly, fractions *D* (Table 2) were analysed for their content of glycerol and fatty acids. Fractions *D* from cow 1 and cows 2–7 had fatty acid:glycerol ratios of 3·2:1 and 2·85:1 respectively, indicating that they consisted essentially of triglycerides; glycerol was detected, but only in trace amounts, in fractions *B* and *E*. Free acidity was found titrimetrically in fraction *D* from cow 1, though none was present in the corresponding fraction of the lipids from cows 2–7.

The iodine values of the total recovered lipids, calculated from the iodine values of the individual (pooled) lipid fractions (see Table 2) were 94·7 and 109·0 respectively as compared with 99·0 and 115·5 determined directly before chromatography on the lipids of cow 1 and cows 2–7 respectively.

After the completion of the chromatography the columns were each washed with 1 l. of ether–methanol (1:1, v/v); in neither case was more than 25 mg. of dark-yellow, apparently oxidized, oil removed accompanied by very fine particles of silica. Since surprisingly small amounts of phosphorus-containing lipids were found in the plasma extracts, the protein residue in each case was exhaustively extracted with hot chloroform–methanol (1:1, v/v). These extracts yielded about 0·5 g. of soluble matter, of which only about 10% was subsequently soluble in chloroform alone. This chloroform-soluble material was a viscous, dark-brown oil having a phosphorus content of approximately 0·1%.

As already indicated, the major component (β-carotene) of the coloured substances present in the plasma lipids was eluted along with the main sterol ester fraction, and a second yellow band accompanied the sterols. Among other minor coloured zones observed was a pale-pink band which ran ahead of the β-carotene zone, but which became very diffuse during elution of the column with 1% ether in light petroleum. A pale-green band remained almost at the top of the column until it was eluted with ether–methanol.

DISCUSSION

The method of chromatography employing light petroleum–ether mixtures for the elution of adsorbed lipids from silicic acid columns (Fillerup & Mead, 1953*a*, *b*) was chosen in preference to the procedure of Borgström (1952*b*), in which benzene is a component of the eluting solvent, since we wished to avoid any possible interference with spectrophotometric analysis in subsequent studies of fatty acid composition. It is noteworthy that laboratory preparations of silica gel proved to be unsatisfactory, again demonstrating the variability of this material as an adsorbent (cf. Lovern, 1956).

Though it is not claimed that completely homogeneous classes of compounds were obtained chromatographically, it is evident from the analytical

data that the sterol esters, glycerides, free sterols and phospholipids were separated from each other. The Liebermann–Burchard reaction showed that only those column fractions which were pooled to give lipid fractions *B* and *E* (Table 2) contained cholesterol, and quantitative studies showed that all the cholesterol in fractions *B* was esterified, whereas that in fractions *E* was free. Fractions *C*, which represented only a very small proportion of the total lipids, contained, *inter alia*, an esterified sterol or sterols which reacted with the Liebermann–Burchard reagents. Triglycerides were present almost exclusively in fractions *D*. Organic phosphorus was found for the most part in fractions *F*, which doubtless also contained non-lipid material.

The fatty acid composition of the sterol esters and triglycerides from cows 2–7 (see Lough *et al.* 1957) will be discussed in the following paper. An investigation to establish the nature of the sterols, other than cholesterol, which may be present in fractions *B*, *C* and *E*, is being undertaken; it is possible that higher aliphatic alcohols, such as were found by Lovern (1956) in the lipids of haddock flesh, may also be present.

In Table 3 the component lipids extracted from the bulked sample of plasma of the six pasture-fed, lactating animals are compared with those calculated from the results of Kelsey & Longenecker (1941), who obtained their lipids from six similar cows. The extraction procedure used to isolate the lipids was apparently similar in both cases though, as already indicated, the subsequent analysis was carried out by totally different methods. Both our results and those of Kelsey & Longenecker (1941) serve to emphasize the preponderance of sterol (cholesterol) esters in the plasma lipids of the lactating cow and the relatively small proportions of triglycerides and free sterols which are present. Whereas Channon & Collinson (1929) could not detect free fatty acids in the plasma lipids of lactating cows, our preparations did contain small amounts of acidic components, though much less (especially in the lipids of cows 2–7) than were found by Kelsey & Longenecker (1941), who reported the presence of some 10% (mainly palmitic, oleic and linoleic acids) in their material. These free acids may have been derived, as the work of Schaible (1932) suggests, from the decomposition of phospholipids during the extraction procedure.

Table 3. *Analysis of lipids extracted from the plasma of lactating, pasture-fed cows*

Figures are expressed as percentage by wt. of total lipids.

	Present work (cows 2–7) (%)	Kelsey & Longenecker (1941) (%)
Sterol (cholesterol) esters	79·8	54·1
Triglycerides	7·5	9·6
Sterols (cholesterol)	6·4	11·9
Phospholipids	3·8*	13·8
Free fatty acids	Traces	10·6
Unidentified lipids†	2·5	—

* Includes non-lipid material.
† Fractions *A* and *C* (Table 2).

Though we were not primarily interested in the isolation of phospholipids, it is noteworthy that the amounts of lipid phosphorus reported by Kelsey & Longenecker (1941) and by other workers, e.g. Maynard, McCay, Ellis, Hodson & Davis (1938), were considerably greater than those found in either of our lipid extracts. In our work the ethanol–ether extracts were concentrated rapidly under reduced pressure on a hot-water bath (temperature about 80°) and it is possible that some phospholipids were thereby rendered insoluble in light petroleum (cf. Ellis & Maynard, 1937); another possibility, perhaps more likely, is that phospholipids remained in the aqueous ethanol after extraction with light petroleum. In further studies this will be investigated in more detail, though it may be mentioned that, with essentially the same procedure as we have used for cow-plasma lipids, the plasma lipids of lactating sheep were found to contain 17–25% of phospholipids (Garton & Duncan, unpublished work).

SUMMARY

1. Lipids, prepared by ethanol–ether extraction of the plasma of lactating cows, were extracted with light petroleum. The lipids soluble in light petroleum were subjected to chromatography on silicic acid columns.

2. Fractions were obtained which consisted essentially of sterol esters (the main component of the extracts), of triglycerides and of free sterols; other smaller fractions consisted of unidentified lipids and of phospholipids which were accompanied by non-lipid material.

We wish to thank Mr J. V. Evans for taking the blood samples and Dr A. K. Lough for the cholesterol and glycerol analyses.

REFERENCES

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Borgström, B. (1952*a*). *Acta physiol. scand.* **25**, 101.
Borgström, B. (1952*b*). *Acta physiol. scand.* **25**, 111.
Channon, H. J. & Collinson, G. A. (1929). *Biochem. J.* **23**, 663.
Clément, G., Clément-Champougny, J. & Louedec, A. (1954). *Arch. Sci. physiol.* **8**, 233.
Desnuelles, P., Naudet, M. & Rouzier, J. (1948). *Biochim. biophys. Acta*, **2**, 561.

Ellis, G. & Maynard, L. A. (1937). *J. biol. Chem.* **118**, 701.
Fillerup, D. L. & Mead, J. F. (1953*a*). *Proc. Soc. exp. Biol., N.Y.*, **83**, 574.
Fillerup, D. L. & Mead, J. F. (1953*b*). *Atomic Energy Project Report*, 251. University of California, Los Angeles.
Folley, S. J. (1956). *The Physiology and Biochemistry of Lactation*. Edinburgh: Oliver and Boyd.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1944). *Biochem. J.* **38**, 65.
Kelsey, F. E. (1939). *J. biol. Chem.* **130**, 187.
Kelsey, F. E. & Longenecker, H. E. (1941). *J. biol. Chem.* **139**, 727.
Korn, E. D. (1955). *J. biol. Chem.* **215**, 1.
Lambert, M. & Neish, A. C. (1950). *Canad. J. Res.* **28**, 83.
Lough, A. K., Garton, G. A. & Duncan, W. R. H. (1957). *Biochem. J.* **65**, 31P.
Lovern, J. A. (1956). *Biochem. J.* **63**, 373.
Maynard, L. A., McCay, C. M., Ellis, G. H., Hodson, A. Z. & Davis, G. K. (1938). *Mem. Cornell agric. Exp. Sta.* no. 211.
Mead, J. F. & Fillerup, D. L. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 449.
Morton, R. A. (1942). *The Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes*, 2nd ed. London: Adam Hilger.
Savary, P. & Desnuelles, P. (1956). *Biochim. biophys. Acta*, **21**, 349.
Schaible, P. J. (1932). *J. biol. Chem.* **95**, 79.
Schoenheimer, R. & Sperry, W. M. (1934). *J. biol. Chem* **106**, 745.
Trappe, W. (1938). *Biochem. Z.* **296**, 180.

Blood Lipids

2. PLASMA LIPIDS OF THE LACTATING COW: FATTY ACID COMPOSITION OF THE STEROL ESTERS AND TRIGLYCERIDES

BY A. K. LOUGH AND G. A. GARTON
Rowett Research Institute, Bucksburn, Aberdeenshire

(*Received* 13 *March* 1957)

Since the triglycerides and possibly the sterol esters of blood plasma are involved in milk-fat formation in the cow (see review by Folley, 1956), it is of considerable biochemical interest to study the fatty acid composition of these classes of lipid, especially in relation to the fatty acid composition of dietary, depot and milk fats. The only information concerning the component fatty acids of the plasma lipids of lactating cows is that obtained by Kelsey & Longenecker (1941), who determined by ester fractionation the composition of the triglycerides, sterol esters and free fatty acids. However, as discussed by Lovern (1956) and Garton & Duncan (1957), it seems doubtful whether the cholesterol esters and triglycerides were effectively separated from each other by the procedure employed, namely selective enzymic hydrolysis of the glycerides. In this laboratory we preferred to use the more recently developed technique of chromatography on silicic acid (Fillerup & Mead, 1953) to separate the sterol esters and triglycerides from the plasma lipids of lactating cows (Garton & Duncan, 1957), and in this paper we describe the determination, on a semi-micro scale, of the fatty acid composition of these classes of lipid by means of reversed-phase partition chromatography. A preliminary account of this work has been published (Lough, Garton & Duncan, 1957).

EXPERIMENTAL

Preparation of fatty acids

The sterol esters and triglycerides used were those prepared by chromatography on silicic acid by Garton & Duncan (1957) from the pooled plasma lipids of six lactating, pasture-fed Ayrshire cows. The sterol esters obtained chromatographically were hydrolysed with ethanolic KOH to yield pale-yellow, liquid fatty acids having an iodine value (Trappe, 1938) of 189·5; the triglyceride fraction was similarly treated to give almost colourless, solid fatty acids of iodine value 36·3.

Estimation of polyethenoid fatty acids by alkali isomerization

Samples of the sterol-ester fatty acids and the glyceride fatty acids were examined according to the method of Hilditch, Patel & Riley (1951), which is applicable to linoleic and linolenic acids but is not applicable in the presence of arachidonic acid in significant amounts. The presence of arachidonic acid is indicated by $\lambda\lambda_{max.}$ 301 and 315 mμ; none was detected in the glyceride fatty acids, but appreciable amounts were present in the sterol-ester fatty acids. Accordingly, extinction coefficients for this acid (not available to us in a pure form) were derived from the data given by Brice, Swain, Herb, Nichols & Riemenschneider (1952), who carried out alkali-isomerization studies with arachidonic acid under similar conditions to those used by Hilditch *et al.* (1951). These values, after isomerization at 180° for 60 min., were taken as $E^{1\%}_{1\,cm.}$ at 315 mμ = 201, and

$E^{1\%}_{1\,cm.}$ at 234 mμ = 515. To allow for the contribution of arachidonic acid to the absorption at 268 mμ after isomerization at 170° for 15 min., the value $E^{1\%}_{1\,cm.}$ = 644 was employed. This value can be considered as an approximation since it was derived by multiplying the value $E^{1\%}_{1\,cm.}$ at 268 mμ = 620 after isomerization at 180° for 15 min. (Brice *et al.* 1952) by the factor 532/512, these being the extinction coefficients at 268 mμ for linolenic acid isomerized for 15 min. at 170° and 180° respectively (Hilditch *et al.* 1951). The proportions of linoleic, linolenic and arachidonic acids thus found are given in Table 1. It should be noted that no account has been taken of the possible presence of *trans* isomers (cf. Shorland, 1955), which may affect extinction coefficients. Traces of a conjugated isomer of linoleic acid were observed in the fatty acids of the sterol esters when they were examined spectrophotometrically before alkali isomerization.

Reversed-phase partition chomatography

The method of chromatography used was essentially that described by Howard & Martin (1950) for the separation of mixtures of higher, saturated *n*-fatty acids in which successively increasing proportions of acetone in water are used to elute the acids individually from a column of Hyflo Super-Cel treated with dichlorodimethylsilane and liquid paraffin. As used on a semi-micro, quantitative scale in this Laboratory, the method has recently been described in detail (Garton & Lough, 1957). Except when stated, the separations were done on columns 30 cm. × 1·5 cm., with acetone–water mixtures containing, successively, 40, 45, 50, 53, 55, 58, 60, 63, 65, 68, 70, 73, 75 and 78% (v/v) acetone. Recoveries of acids from the columns were 95–98%.

RESULTS

Sterol-ester fatty acids

To determine the molecular species of fatty acids present in the mixture, a sample was hydrogenated, as described by Popják & Tietz (1954), to an iodine value of < 1·0, and 26 mg. of the saturated acids was chromatographed. The resulting elution pattern, given in Fig. 1, shows that myristic, palmitic, stearic and arachidic acids were present. The proportions of each component, calculated from the amount of standard alkali required to neutralize the fatty acid present in each peak, are given in Table 2

A sample of the unhydrogenated fatty acids (126 mg.) was chromatographed on a column 40 cm. × 3·0 cm., which permitted the recovery of fatty acid fractions for further examination. The separation, illustrated in Fig. 2, shows that most of the acids were eluted in peaks *A* and *B*, followed by peaks *C*, *D* and *E* of much smaller magnitude; on a molar basis, peaks *A*, *B*, *C*, *D* and *E* represented 38·6, 49·8, 8·7, 2·1 and 0·8% respectively of the total fatty acids.

The column fractions 1–91 (peak *A*) and 92–131 (peak *B*) were collected and the component fatty

Table 1. *Proportions (%, w/w) of polyethenoid components found spectrophotometrically after alkali isomerization of the total fatty acids of the sterol esters and triglycerides*

	Source of fatty acids	
Acid	Sterol esters	Triglycerides
Linoleic	35·2*	1·9
Linolenic	30·2	0
Arachidonic	8·4	0

* Including traces of a conjugated isomer.

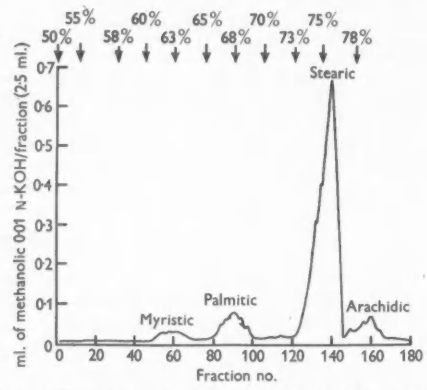


Fig. 1. Chromatography of the hydrogenated fatty acids (26 mg.) of the sterol esters. Arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone–water mixtures used as eluent.

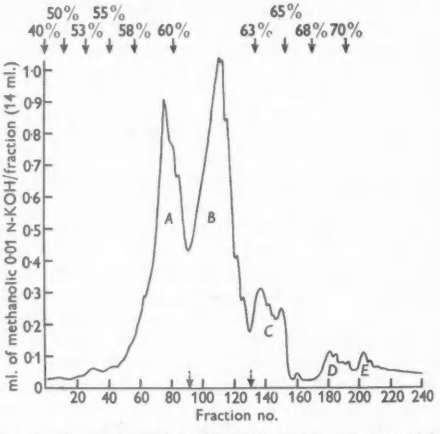


Fig. 2. Chromatography of the fatty acids (126 mg.) of the sterol esters. Uniform arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone–water mixtures used as eluent. Broken arrows indicate the fractions (1–91 and 92–131) which were pooled to yield the acids contributing to peaks *A* and *B* respectively. For composition of peaks *A*, *B*, *C*, *D* and *E* see text.

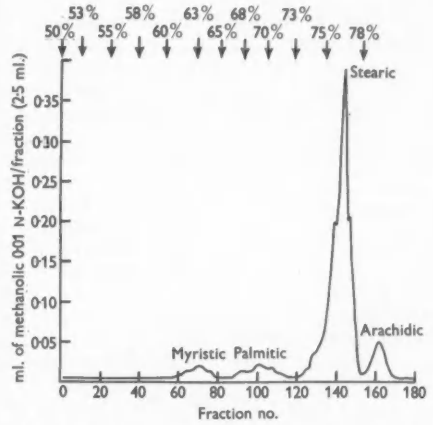


Fig. 3. Chromatography of the hydrogenated fatty acids (11 mg.) of peak *A* (Fig. 2). Arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone–water mixtures used as eluent.

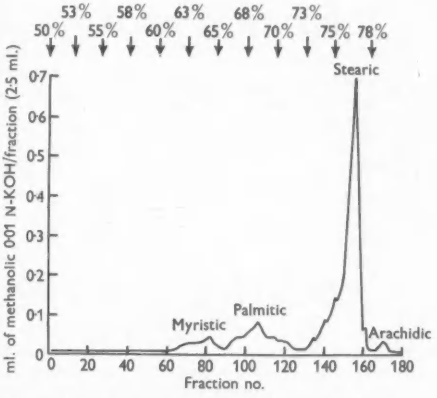


Fig. 4. Chromatography of the hydrogenated fatty acids (18 mg.) of peak *B* (Fig. 2). Arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone–water mixtures used as eluent.

Table 2. *Molar percentage of fatty acids found after hydrogenation*

	Source of fatty acids		
Acid	Total acids of sterol esters	Peak *A* (Fig. 2)	Peak *B* (Fig. 2)
Myristic	4·4	2·2	7·2
Palmitic	12·8	6·8	17·3
Stearic	76·0	82·8	71·8
Arachidic	6·8	8·2	3·7

acids recovered. The iodine values (approximate owing to small amount of acids available) were 247 and 146 for the acids from peaks *A* and *B* respectively. After hydrogenation to iodine values $< 1{\cdot}0$, 11 mg. of acids (peak *A*) and 18 mg. of acids (peak *B*) were subjected to chromatographic examination, and gave the elution patterns shown in Figs. 3 and 4 and the proportions of fatty acids given in Table 2.

It will be observed that the hydrogenated acids from each peak consisted mainly of stearic acid, together with small proportions of myristic, palmitic and arachidic acids. From a consideration of the elution characteristics of unsaturated fatty acids (Crombie, Comber & Boatman, 1955) and the knowledge that no acid of chain length greater than 20 carbon atoms was present in the mixture (Fig. 1), it was concluded that the palmitic and arachidic acids found in peaks *A* and *B* (Fig. 2) represented palmitoleic and arachidonic acids present in the original fatty acids; the myristic acid found in peaks *A* and *B* must have been present before hydrogenation, since no peak corresponding to an unsaturated C_{14} acid was found. It was concluded that the stearic acid in the hydrogenated acids of peak *A* was derived from linolenic acid and that in the hydrogenated acids of peak *B* from linoleic acid [cf. the iodine values of the acids in each peak and the findings of Crombie *et al.* (1955) that linolenic and linoleic acids are separable from each other]. That these conclusions were justified will be seen when the proportions of linoleic and linolenic acid, estimated chromatographically to be present in the total acids, are compared with the proportions found spectrophotometrically after alkali isomerization (see Table 3).

The proportions of the acids found, after hydrogenation, in peaks *A* and *B* show that whereas more of the arachidonic acid present in the original mixture was eluted in peak *A* than in peak *B*, more myristic and palmitoleic acids were present in peak *B* than in peak *A*. Thus some degree of resolution of this complex mixture was achieved, although the overwhelming abundance of linoleic and linolenic acids may have affected the elution behaviour of other components (cf. Lovern, 1956).

The proportion of each component present in the total acids of the sterol esters (Table 3) was calculated from the proportions given in Table 2 and the proportions of the components of peaks *C*, *D* and *E* (Fig. 2). No allowance was made for the very small changes in molecular proportions resulting from hydrogenation of the unsaturated acids.

From their elution behaviour it was concluded that peak *C* comprised oleic and palmitic acids, peak *D* stearic acid and peak *E* arachidic acid. The composition of peak *C* was deduced as follows, all proportions, unless otherwise stated, being molar

Table 3. *Molar percentage of fatty acids contributed by peaks A–E (Fig. 2) to the total fatty acids of the sterol esters*

Acid	Peak *A*	*B*	*C*	*D*	*E*	Total acids
Myristic	0·8	3·6	—	—	—	4·4
Palmitic	—	—	1·6	—	—	1·6
Stearic	—	—	—	2·1	—	2·1
Arachidic	—	—	—	—	0·8	0·8
Palmitoleic	2·6	8·6	—	—	—	11·2
Oleic	—	—	6·1	—	—	6·1
Linoleic	—	35·8	—	—	—	35·8 (36·4)*
Linolenic	32·0	—	—	—	—	32·0 (32·3)*
Arachidonic	3·2	1·8	(1·0)†	—	—	6·0 (6·6)*

* Molar percentage of total acids, calculated from the figures given in Table 1.
† Not accounted for by chromatographic results, but attributed to arachidonic acid (see text).

percentages of the total fatty acids. The contribution (1·6 %) of the palmitic acid present in peak *C* was derived by subtracting the contribution (11·2 %) of the palmitic acid of the hydrogenated acids of peaks *A* and *B* from the palmitic acid content (12·8 %) of the total hydrogenated fatty acids. The contribution (6·1 %) of the oleic acid of peak *C* was derived by subtracting the contribution (67·8 %) of the stearic acid in the hydrogenated acids of peaks *A* and *B* plus the stearic acid contribution (2·1 %) of peak *D* from the stearic acid content (76·0 %) of the total hydrogenated fatty acids. Thus together the palmitic and oleic acids in peak *C* contributed 7·7 %, compared with 8·7 % calculated on the basis of the titrations during chromatography, leaving 1 % of acids not accounted for. Experimental error undoubtedly accounted for this minor deficiency in the complete analysis. However, a discrepancy of 1 % was found between the molar proportion of arachidic acid (6·8 %) found after hydrogenation of the total acids and the sum of the arachidic acid contributions of the hydrogenated acids in peaks *A* and *B* (5·0 %), together with that in peak *E* (0·8 %). We therefore feel justified in attributing this 1 % to arachidonic acid.

The iodine value (192·4) of the total fatty acids calculated from the results of the chromatographic analysis compares favourably with that obtained by direct determination (189·5).

Triglyceride fatty acids

The fatty acids were chromatographed before and after hydrogenation, 21 and 16 mg. respectively being used; the elution patterns are illustrated in Fig. 5.

It will be observed from the chromatogram of the hydrogenated acids that four molecular species of acids (C_{14}, C_{16}, C_{18} and C_{20}) were present; the molar contribution of myristic, palmitic, stearic and arachidic acids to the total mixture was 6·4, 37·1, 53·6 and 2·9 % respectively. No peak corresponding

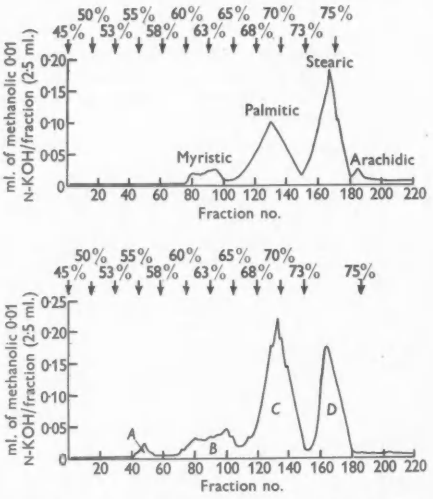


Fig. 5. Chromatography of the fatty acids of the triglycerides. Upper chromatogram, 16 mg. of the hydrogenated acids; lower chromatogram, 21 mg. of the unhydrogenated acids; for description of peaks *A–D*, see text. Arrows indicate progressive changes in the proportions (v/v) of acetone in the acetone–water mixtures used as eluent.

Table 4. *Composition of the glyceride fatty acids*

Acid	Molar percentage
Myristic	4·6
Palmitic	29·9
Stearic	30·6
Myristoleic	1·8
Palmitoleic	7·2
Oleic	21·5
Linoleic	1·5
Gadoleic	2·9

to a C_{20} acid was observed in the chromatogram of the unhydrogenated acids and, since no arachidonic acid was detected spectrophotometrically (Table1), it was concluded that the arachidic acid found after hydrogenation was derived from the C_{20} mono-unsaturated acid, gadoleic acid. Before hydrogenation, a peak corresponding to lauric acid was observed, but since it disappeared on hydrogenation it was attributed to the C_{14} mono-unsaturated acid, myristoleic acid. The molar contribution to the total fatty acids of peaks *A*, *B*, *C* and *D* (Fig. 5) was 1·8, 13·3, 51·4 and 33·5 % respectively. Calculation of the final composition of the glyceride fatty acids (Table 4) thus entailed simple subtractions, due allowance being made (in peak *B*) for the proportion of linoleic acid found spectrophotometrically to be present (Table 1). The iodine value of the total acids was calculated to be 34·3, compared with the value of 36·3 found by direct determination.

DISCUSSION

Chromatography

The reversed-phase partition chromatographic method of Howard & Martin (1950) for the quantitative separation of milligram quantities of lauric, myristic, palmitic and stearic acids was extended by Silk & Hahn (1954) and Popják & Tietz (1954) to include the even-numbered, *n*-fatty acids C_{10}–C_{24}, by Hougen (1955) to include the odd-numbered, *n*-fatty acids C_7, C_9 and C_{11}, and further by Garton & Lough (1957) to embrace all the saturated *n*-fatty acids C_8–C_{20}. Crombie *et al.* (1955) applied the method to the analysis of fairly simple mixtures of saturated and unsaturated fatty acids of plant origin by running chromatograms before and after oxidative decomposition of the unsaturated components with alkaline potassium permanganate. As in the reversed-phase chromatographic procedure of Boldingh (1953), we preferred to run chromatograms of mixed acids before and after hydrogenation of the unsaturated components (cf. Popják & Tietz, 1954), not only because the molecular species of acids present in the hydrogenated mixture are thus identifiable, but also because the alkaline permanganate treatment brings about some destruction of saturated fatty acids; in preliminary studies we confirmed that partial oxidative degradation of *n*-fatty acids C_{14} and lower occurred as reported by Crombie *et al.* (1955).

As exemplified by the analyses reported in this paper it seems that the method involving hydrogenation of the unsaturated fatty acids can be commended as a semi-micro, quantitative analytical technique for the investigation of lipids of animal origin, especially when myristic acid and saturated acids of lower chain-length are present. The assumptions made in the evaluation of the chromatograms are few and, when polyethenoid components are involved, their presence and proportions can be determined by complementary alkali-isomerization studies. We therefore feel that the analyses presented are comparable in their accuracy with those determined on fatty acid mixtures by the ester-fractionation technique (cf. Garton & Lough, 1957) which, of course, requires far more starting material than is readily available from small tissue samples and blood.

Sterol-ester fatty acids

The most striking feature of these acids is their very high iodine value (189·5) which is reflected in their content of the polyethenoid components linoleic, linolenic and arachidonic acids. Schaible (1932), who isolated lipid fractions rich in cholesterol esters from the plasma of lactating and non-lactating cows, found that the iodine values of the acids were about 150, whereas that calculated from the analysis of Kelsey & Longenecker (1941) is 155·9 for the acids present in their crude concentrate of cholesterol esters. In the analysis reported by Kelsey & Longenecker (1941) linoleic, linolenic and arachidonic acids comprised 61·7, 9·2 and 2·3 % (mol.) respectively of the total fatty acids, whereas in our preparation the two C_{18} unsaturated acids were present in nearly equal proportions [35·8 and 32·0 % (mol.) respectively], together with 6·0 % (mol.) of arachidonic acid.

Cholesterol esters differ from ox depot fat and plasma triglycerides (Table 5) in their content of polyethenoid fatty acids. It is noteworthy that the components of these esters must be derived from different sources, the cholesterol being produced endogenously (there being no dietary source), and the C_{18} di- and tri-unsaturated fatty acids arising from the diet, of which pasture grasses are a good source (see Hilditch, 1956). It is thus possible that preferential esterification of these acids with cholesterol takes place during, or subsequent to, their absorption from the small intestine, though the formation of cholesterol arachidonate presumably involves the esterification of arachidonic acid produced *in vivo* from linoleic acid, as was shown by Mead, Steinberg & Howton (1953) to occur in the rat. However, it is probable that only a small proportion of the dietary polyunsaturated fatty acids reaches the small intestine, since the work of Shorland, Weenink & Johns (1955) strongly suggests that unsaturated fatty acids are hydrogenated to a considerable extent in the reducing environment of the rumen.

Though it may be only fortuitous, the molar proportion of *total* C_{18} unsaturated fatty acids in the sterol esters (73·9 %) resembles (see Table 5) the proportion (78·5 %) reported in the fatty acids of mixed pasture grasses by Jasperson & Burke (cited

Table 5. *Molar percentage of the fatty acids in ox depot fat, milk fat, plasma sterol esters and triglycerides of lactating cows and in the lipids of pasture grasses*

Acid	Typical milk fat (Hilditch & Jasperson, 1943)	Typical depot fat (Hilditch & Longenecker, 1937)	Plasma triglycerides (present work)	Plasma sterol esters (present work)	Mixed pasture grasses (Jasperson & Burke; cited by Hilditch, 1956)
Saturated					
Short-chain, C_4–C_{12}	21·7	0·2	—	—	2·9
Myristic	9·6	3·7	4·6	4·4	3·3
Palmitic	23·4	26·5	29·3	1·6	9·4
Stearic	9·7	23·1	30·6	2·1	1·5
Arachidic	0·6	0·7	—	0·8	0·7
Unsaturated					
$C_{10}+C_{12}$	0·5	—	—	—	0·3
Myristoleic	1·0	0·5	1·8	—	0·4
Palmitoleic	2·1	2·6	7·2	11·2	3·0
Oleic	28·6	40·4	21·5	6·1	} 78·5‡
Linoleic	1·8	1·8	1·5	35·8	
Linolenic	—	—	—	32·0	
C_{20}–C_{22}	1·0	0·5*	2·9†	6·0*	—

* As arachidonic acid.
† As gadoleic acid.
‡ Comprising approx. 50% of linolenic acid, together with linoleic and oleic acids.

by Hilditch, 1956) and by Shorland (1944), who found 76·5%, composed of about equal amounts of linoleic and linolenic acids, in the fatty acids of cocksfoot (*Dactylis glomerata*).

Triglyceride fatty acids

As shown in Table 5, the fatty acid composition of the plasma triglycerides resembles that of depot fat in that the major components are stearic, palmitic and oleic acids, together with smaller amounts of myristic, myristoleic, palmitoleic, linoleic and C_{20} unsaturated acids. The absence of linolenic and arachidonic acids is especially noteworthy in view of the amounts found in sterol–ester combination.

Whereas the saturated fatty acids C_4–C_{16} of ruminant milk fat can be synthesized in the udder (Popják, French, Hunter & Martin, 1951; Cowie *et al.* 1951), it seems that stearic acid and oleic acid (the major component of milk glycerides, see Table 5) must be derived from plasma triglycerides, though the mechanism of incorporation of these acids into milk glycerides is obscure. Hilditch (1952) has suggested that the replacement of oleic acid of plasma triglycerides by saturated acids (especially the shorter-chain acids) may take place, though it is also possible that the oleic acid may, in some way, become available for incorporation into glycerides, being synthesized *de novo* in the secretory tissue of the udder. Such a possibility is suggested by our finding that the oleic acid content of the plasma triglycerides is only about one-half of that found in depot fat, which is presumably the source of much of the plasma triglycerides in the lactating cow. In this connexion it should be noted that, as a result of feeding tritium-labelled stearic acid (as free acid or triglyceride) to lactating goats and cows, Glascock, Duncombe & Reinus (1956) concluded that not more than 25% of milk glyceride fatty acids are derived from the dietary lipids.

It is relevant to note that when groundnut, rape, soya-bean and linseed oils were fed to lactating cows (Hilditch & Sleightholme, 1930; Hilditch & Thompson, 1936), the amount of polyethenoid C_{18} fatty acids of the milk fat showed no significant increase, in spite of the high linoleic and linolenic acid content of the oils. Recently Mayhead & Barnicoat (1956) found that the iodine value of bovine milk fat increased by 5·7 units during partial inanition, and when 16 oz. of groundnut oil (iodine value, 96·2)/cow/day was administered during partial inanition the iodine value of the milk fat increased by only 2·3 units. It was expected that the oil feeding would result in a more pronounced rise in unsaturation than that occurring during partial inanition alone, since the unsaturated oil was expected to have a sparing action on the utilization of depot fat. In the light of our findings and those of Shorland *et al.* (1955), the results of Hilditch & Sleightholme (1930), Hilditch & Thompson (1936) and Mayhead & Barnicoat (1956) may well be explained by the partial hydrogenation of polyunsaturated dietary acids followed by esterification of the remaining polyunsaturated acids with cholesterol, which results in their unavailability for incorporation into milk glycerides.

SUMMARY

1. The reversed-phase partition chromatographic method of Howard & Martin (1950) has been extended to the semi-micro, quantitative analysis of the fatty acids of the sterol esters and triglycerides from the plasma of pasture-fed, lactating cows.

2. The sterol-ester fatty acids were characterized by their high content of polyunsaturated components (linoleic, linolenic and arachidonic acids), whereas the triglycerides resembled depot fat in the nature of the component fatty acids, though the proportion of oleic acid was considerably lower in the plasma glycerides than in depot fat.

3. The fatty acid composition of the two classes of plasma lipid is discussed in relation to that of dietary lipids and milk glycerides. It is suggested that dietary polyunsaturated fatty acids which escape hydrogenation in the rumen may be preferentially esterified with cholesterol during, or subsequent to, their absorption from the alimentary tract.

We wish to thank Mr W. R. H. Duncan for helpful discussion and Mr N. I. Lovie for his technical assistance.

REFERENCES

Boldingh, J. (1953). *Int. Conf. Biochem. Probl. Lipids, Brussels*, p. 64.

Brice, B. A., Swain, M. L., Herb, S. F., Nichols, P. L. jun. & Riemenschneider, R. W. (1952). *J. Amer. Oil Chem. Soc.* **29**, 279.

Cowie, A. T., Duncombe, W. G., Folley, S. J., French, T. H., Glascock, R. F., Massart, L., Peeters, G. & Popják, G. (1951). *Biochem. J.* **49**, 610.

Crombie, W. M. L., Comber, R. & Boatman, S. G. (1955). *Biochem. J.* **59**, 309.

Fillerup, D. L. & Mead, J. F. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 574.

Folley, S. J. (1956). *The Physiology and Biochemistry of Lactation*. Edinburgh: Oliver and Boyd.

Garton, G. A. & Duncan, W. R. H. (1957). *Biochem. J.* **67**, 340.

Garton, G. A. & Lough, A. K. (1957). *Biochim. biophys. Acta*, **23**, 192.

Glascock, R. F., Duncombe, W. G. & Reinus, L. R. (1956). *Biochem. J.* **62**, 535.

Hilditch, T. P. (1952). *Symp. biochem. Soc.* no. 9, p. 63.

Hilditch, T. P. (1956). *The Chemical Constitution of Natural Fats*, 3rd ed. revised, p. 153. London: Chapman and Hall Ltd.

Hilditch, T. P. & Jasperson, H. (1943). *Biochem. J.* **37**, 238.

Hilditch, T. P. & Longenecker, H. E. (1937). *Biochem. J.* **31**, 1805.

Hilditch, T. P., Patel, C. B. & Riley, J. P. (1951). *Analyst*, **76**, 81.

Hilditch, T. P. & Sleightholme, J. J. (1930). *Biochem. J.* **24**, 1098.

Hilditch, T. P. & Thompson, H. M. (1936). *Biochem. J.* **30**, 677.

Hougen, F. W. (1955). *Biochem. J.* **59**, 302.

Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.

Kelsey, F. E. & Longenecker, H. E. (1941). *J. biol. Chem.* **139**, 727.

Lough, A. K., Garton, G. A. & Duncan, W. R. H. (1957). *Biochem. J.* **65**, 31P.

Lovern, J. A. (1956). *Biochem. J.* **63**, 373.

Mayhead, J. W. & Barnicoat, C. R. (1956). *J. Dairy Res.* **23**, 238.

Mead, J. F., Steinberg, G. & Howton, D. R. (1953). *J. biol. Chem.* **205**, 683.

Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.

Popják, G. & Tietz, A. (1954). *Biochem. J.* **56**, 46.

Schaible, P. J. (1932). *J. biol. Chem.* **95**, 79.

Shorland, F. B. (1944). *Nature, Lond.*, **153**, 168.

Shorland, F. B. (1955). In *Progress in the Chemistry of Fats and other Lipids*, vol. 3, p. 315. Ed. by Holman, R. T., Lundberg, W. O. & Malkin, T. London and New York: Pergamon Press.

Shorland, F. B., Weenink, R. O. & Johns, A. T. (1955). *Nature, Lond.*, **175**, 1129.

Silk, M. H. & Hahn, H. H. (1954). *Biochem. J.* **46**, 532.

Trappe, W. (1938). *Biochem. Z.* **296**, 180.